STUDIES ON THE PHOSPHOENOLPYRUVATE-DEPENDENT PHOSPHOTRANSFERASE SYSTEMS IN *Streptococcus mutans* GS5

BY

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Abstract of Dissertation Presented to the Graduate Council
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STUDIES ON THE PHOSPHOENOLPYRUVATE-DEPENDENT
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Streptococcus mutans GS5

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This dissertation is concerned with characterization of the glucose phosphoenolpyruvate (PEP)-dependent phosphotransferase system (glc PTS) in Streptococcus mutans GS5. The PTS is a mode of transport which involves the translocation of a phosphoryl group from PEP to an incoming sugar moiety. It is a complex system which requires up to four proteins, Enzyme I (EI), HPr, Enzyme III (EIII), and Enzyme II (EII), to accomplish the transport and group translocation functions.

The objectives of the present study were (1) to obtain a general overview of the glc PTS, (2) to obtain a more detailed picture of the glc PTS by studying isolated membranes derived from cells of S. mutans, and (3) to study the hierarchy of sugar utilization in S. mutans.

The substrates of the glc PTS in order of declining affinities were found to be glucose, mannose, and 2-deoxyglucose. The evidence for this finding comes from studies of the competitive effects exerted by the above sugars on the transport of the heterologous sugars, from kinetic studies, and from studies on glc PTS-negative mutants.

Isolated membranes derived from cells grown in glucose were prepared using the muralytic enzyme, mutanolysin. These membranes were able to phosphorylate glucose and mannose when supplied with exogenous PEP. They were also able to phosphorylate glucose when the phosphoryl donor glucose-6-phosphate was used (transphosphorylation), thus demonstrating the presence of a functional EII^{glc} in cell-free membranes of the wild-type strain. The presence of EI in these membrane preparations was demonstrated by the phosphoryl exchange reaction between PEP and pyruvate.

Glucose in the growth-medium prevented the induction by lactose of the lac PTS in S. mutans GS5. Thus, glucose is a preferred sugar. Glucose did not appear to repress the induction of the lac PTS in glc PTS-negative cells even though glucose was taken up by these cells. This finding indicates the necessity for a functioning glc PTS for the regulation of lactose uptake. The mutant cells contained wild-type levels of EI but lacked a functioning EII^{glc} as shown by the two phosphoryl exchange reactions. These results suggest that the EII^{glc} is required for the regulation of sugar uptake in S. mutans GS5.

INTRODUCTION AND LITERATURE REVIEW

Streptococcus mutans is the causative agent of dental caries, the most prevalent bacterial disease in humans. This species is divided into a number of serotypes (a-g), however serotype c is most commonly isolated from carious lesions. The growth of this organism is accompanied by the production of lactic acid which causes the demineralization of dental enamel. The resulting cavity is the clinical manifestation of this disease (13,14). Numerous investigators have been studying the physiology of sugar metabolism of S. mutans in order to deduce basic mechanisms of transport and dissimilation and to learn about the genetic basis of the pertinent enzymology. It is hoped that such knowledge could be applied to preventative therapy and thus aid in the elimination of this disease.

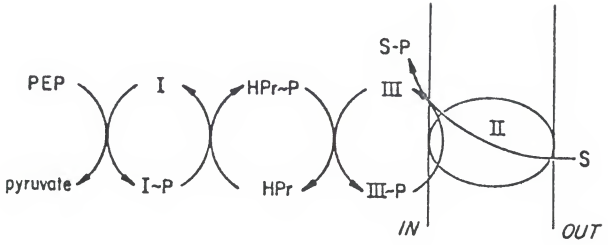
At this stage only limited knowledge exists concerning the transport of sugars into S. mutans. The purpose of this dissertation is to expand our knowledge of a major transport route, the phosphotransferase system, in serotype c strain GS5.

Mechanisms of transport. Carbohydrate transport in bacteria is characterized by three general modes. The first is exemplified by the lactose system in Escherichia coli. The energy required for lactose uptake is derived from proton symport, i.e., lactose is co-transported with a proton, thus dissipating the proton gradient produced during electron transport. The second type of transport utilizes adenosine

5'-triphosphate (ATP) as an energy source and is exemplified by maltose uptake in E. coli. This type of transport is characterized by the requirement for a periplasmic binding protein. One way these two types of sugar accumulations are differentiated is by the loss of transport functions upon osmotic shock in the case of the latter transport system and the retention of such functions in the former system (7). The third mode of transport is characterized by glucose transport in E. coli and lactose transport in Staphylococcus aureus (21). It is termed the phosphoenolpyruvate (PEP)-dependent phosphotransferase system (PTS). This type depends on the energy inherent in the enol configuration of the phosphoryl group of PEP. Since the phosphoryl group is transferred, it falls into the general category of group translocation.

Components of the phosphotransferase system. The two systems which have been studied in the most detail, and after which a standard scheme has been modeled, are the glucose system of E. coli and the lactose system of S. aureus. This model has been developed by the isolation of individual components and their reconstitution in vitro. A general scheme for PTS-mediated sugar transport is outlined in Fig. 1. The phosphoryl group is transferred from PEP to a small molecular-weight protein called HPr. This translocation is mediated by the enzyme termed Enzyme I (EI). EI itself forms a phosphorylated intermediate. From HPr, the phosphoryl group is transferred to a third protein referred to as Enzyme III (EIII). The final steps involve the concomitant transfer of the phosphoryl group from EIII to the sugar as it crosses the cytoplasmic membrane. The permease is referred to as Enzyme II (EII). Both EIII and EII are sugar specific (31,55,77).

Fig. 1. Schematic of the phosphoenolpyruvate-dependent phosphotransferase system (55). I (Enzyme I) and HPr refer to the general phosphoryl carriers found in the cytosol. III (Enzyme III) refers to the sugar specific phosphoryl donor. II (Enzyme II) is the membrane-bound permease.


PEP
I
HPr~P
III
S-P
II
S
pyruvate
I~P
HPr
III~P
IN
OUT

The first two proteins (HPr, EI) are general proteins in that they are involved in the transport of all "PTS sugars" by a given cell (21, 25,78). HPr has been studied in S. aureus and E. coli. In both cases it is a small molecular weight protein; the E. coli HPr is 9600 daltons (1) and the S. aureus HPr is 9000-9200 daltons (77). Its physiological activity is stable to heating at 100 C for several minutes (25,29). From studies of the rates of hydrolysis of phospho-HPr (HPr ~ P) under acid and alkaline conditions, it has been determined that the phosphoryl group is transferred to the N-1 of the imidizole ring of histidine (1,76). This component has been shown to be non-specific since point mutations in the gene coding for HPr produces pleiotropic effects in terms of sugar transport (55,78). Attempts to cross species lines have met with little success, although the E. coli HPr allows low levels (5% of the homologous system) of phosphorylation of thiomethyl-β-D-galactopyranoside (TMG) by the S. aureus PTS enzymes. However, the S. aureus HPr cannot substitute for E. coli HPr when α-methylglucoside (α-MG) is the substrate (77). Durham and Phibbs (9) were unable to demonstrate cross-complementation between Pseudomonas aeruginosa and E. coli. Simoni et al. (77) reported the block occurrred between HPr ~ P and sugar phosphorylation when S. aureus HPr was substituted in the E. coli system. Cords and McKay (6) reported cross-complementation of crude extracts from S. aureus and S. lactis; however, the results are difficult to evluate since a quantitative analysis was not presented.

The EI protein has been isolated from S. Aureus and E. coli. However it has been only partially purified and the failure to attain full purification of this protein has been attributed to its sensitivity to

oxidation (25,30). In S. aureus, the phosphorylated derivative of this enzyme migrates as a single band of 80,000 daltons in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (25). The molecular weight of this enzyme in E. coli and Salmonella typhimurium has been estimated to be 70,000 and 90,000 daltons, respectively (21).

The cytoplasmic localization of EI and HPr was determined by the complete separation of cytoplasmic and membrane fractions (31,77). In S. aureus this was accomplished by ultracentrifiguation (77). The biological activity of HPr and EI has been elucidated mainly in the laboratory of Dr. S. Roseman from studies with E. coli and S. aureus. Kundig and Roseman (30) demonstrated that in the presence of ^{32}P-enolpyruvate, and Mg^{2+}, the rate of the reaction involving the transfer of the phosphoryl group to HPr was directly proportional to EI concentration, whereas HPr demonstrated saturability. This indicated that the catalyst was EI while HPr served as a phosphoryl carrier. The reaction is stoichiometric and dependent on the PEP concentration. Furthermore, determination of equilibrium constants using components isolated in S. aureus demonstrated that the energy inherent in the enolate bond of PEP is essentially maintained in HPr ~ P (76). During this transfer EI forms a transitory phosphorylated intermediate (21, 25). With high concentrations of PEP and pyruvate, an abortive complex is formed and thus the PTS reaction is inhibited. Saier et al. (64) used this observation to develop a reaction to directly assay for the presence of EI; that is, EI catalyzes a phosphoryl exchange between PEP and pyruvate.

The sugar-specific component, EIII, shows more variation among organisms and also among PT-systems within the same organism. This is best illustrated by three systems: the lactose PTS (lac PTS) of S. aureus and the two glucose phosphotransferase systems (glc PTS) of E. coli.

The EIII of the lac PTS in S. aureus was purified from the soluble fraction of an ultracentrifugation of cell extracts (22). Studies showed it to be a trimer composed of three identical sub-units. Various physical techniques such as analytical ultracentrifugation and SDS-PAGE allowed a measurement of 35,700 daltons for the trimer (22). This protein is auto-catalytic; i.e., it catalyzes the transfer of the phosphoryl group from HPr to itself (22). Each sub-unit is capable of accepting a phosphoryl group on a histidyl residue. Hydrolysis under acid and alkaline conditions allowed the determination of the phosphoryl bond to be at the N-3 of the imidizole ring (22,25). The phospho-EIII (EIII ~ P) then is able to donate the phosphoryl group to lactose (76). Experiments with membrane-bound EIII have demonstrated that the phosphorylated form of EIII exhibits lipophilic behavior, whereas the non-phosphorylated does not. Sub-unit exchange was readily demonstrated with the phosphorylated form but not the underivatized form. Also, dissociation of the phosphorylated form by lipophilic agents was observed. It was suggested that physiologically EIII exists as an underivatized, non-phosphorylated trimer and a membrane-bound phosphorylated monomer. It is in the latter form that the EIII has been postulated to be able to intercalate in the membrane and to donate the phosphoryl group to the incoming sugar (25).

As stated, in E. coli there are two different glc PT-systems. One has been characterized as a high-affinity system and is able to phosphorylate the glucose analogue, α-MG, as well as glucose. This system, similar to the lactose system of S. aureus, features a "soluble" EIII. Unlike $EIII^{lac}$, the phosphoryl protein link is via an acyl group (21,25). Its apparent molecular weight is 20,000 (25). As will be described below, $EIII^{glc}$ may have a regulatory role (73).

In contrast to the latter two systems is the low-affinity glucose system of E. coli. This system has been shown to transport glucose and its anomers, mannose and fructose. In addition, it transports the glucose analogue 2-deoxyglucose (2-DG) as well as being responsible for the accumulation of the acetyl and N-acetyl derivatives of glucosamine and mannosamine (30,55). This EIII is an integral membrane protein which is complexed with the translocating component, EII. It has been isolated by Kundig and Roseman (31) and shown by isoelectric focusing to be composed of three proteins each of which showed EIII activity, albeit each with differeing specificities. One band had EIII activity with mannose, a second was specific for fructose and the third was specific for glucose (31).

The soluble components have been isolated and well characterized. Their biological activities have been established through reconstitution. Furthermore, since the phosphoryl addition involves sequential steps, the order as well as the individual proteins involved have been established through the use of a ^{32}P-probe and the isolation of the intermediates by electrophoresis (30) or gel filtration chromatography (76). The transport step and the concomitant phosphoryl transfer to

the sugar is more poorly understood. Transport requires an intact membrane and conversely membrane proteins require lipid matrices to function; thus dissection of this step is a more formidable task.

Various EII's have been isolated and along with EIII~P have been shown to complete the phosphoryl transfer to the sugar. Among the EII's that have been isolated thus far is the EII from the low-affinity glucose system in E. coli (31) and the EII^{lac} of S. aureus (77).

The E. coli EII^{glc} was isolated using a butanol/urea extraction technique. It was shown to have biological activity in the presence of phosphatidylglycerol and the requirement for this phospholipid was stringent (31). This observation was surprising since this is a minor lipid within the E. coli membrane (21).

EII^{lac} was purified by using a combination of agents including sodium deoxycholate, Tween 40 and Triton X-100 (77). Lipid-free EII could be obtained by sucrose-gradient centrifugation and biological activity was detected in the presence of the other PTS components if a lipophilic environment is created with a detergent such as Triton X-100 (25).

Identification of products of phosphotransferase system activity. The definitive assignment of functional phosphotransferase systems involves isolating and identifying the derivatized sugar(s) as product(s). Reconstitution of the S. aureus lac PTS results in the formation of TMG-6-P (77), and lactose-P (78) where the phosphoryl group is esterified at the C6 position of the β-D-galactopyranosyl moieties. The E. coli glucose systems form glucose-6-phosphate (30,55), a direct intermediate in glycolysis. Fructose is phosphorylated at C1 (55).

Other phosphotransferase systems for sugar uptake. Many systems for sugar transport have been investigated to date. Most of these systems conform to the model discussed in the preceding section. However, a few have been found to diverge from the accepted scheme. For illustrative purposes two will be discussed in this section.

The obligate aerobe, P. aeruginosa, contains an unusual PTS for fructose utilization. Only two components have been identified in this system, one membrane bound and one soluble. The soluble protein appears to be similar to EI in terms of molecular weight; however, unlike EI, this may be a peripheral membrane protein since membranes alone have residual PTS activity (9). On the other hand, this residual activity may represent cytoplasmic contamination of the membrane preparation used in the study cited. The present study of S. mutans GS5 will present a similar finding with regard to the glc PTS (see RESULTS).

Another interesting divergence from a better known system is the inducibility of these two components in the pseudomonad. In E. coli, both HPr and EI are constitutive. However, the relative levels are enhanced when glucose is present (53). The membrane components (EII, EIII) vary also with the composition of the medium. However, the relative levels of the sugar-specific components allow for the conclusion that the E. coli low affinity glucose system is constitutive and the high affinity system is inducible (21). The sugar specific components (EII and EIII) of the S. aureus lac PTS are inducible whereas the equivalent components for glucose transport are constitutive (21). A different pattern is observed in P. aeruginosa. Both factors are inducible (9). This may be related to the presumably secondary role the PTS plays in this organism.

The occurrence of the PTS is directly related to the metabolism of the organism. In general, strict aerobes do not have a PTS. There are a number of exceptions such as P. aeruginosa. Also, heterofermentative genera do not transport sugars via a PTS. This is presumably due to the stoichiometric relationship between the fermented sugar and the generation of PEP (21,54). Again P. aeruginosa is an obvious exception to this generalization. Durham and Phibbs (9) have proposed that after being brought in by a PTS, a putative kinase is involved in phosphorylating fructose. Thus, fructose plus PEP results in fructose-1-phosphate which then accepts a second phosphoryl group from ATP to form fructose-1,6-diphosphate. Generally, organisms which have phosphotransferase systems are either anaerobes or facultative anaerobes. These organisms utilize the Embden-Myerhof pathway and thus generate two moles of PEP for every mole of sugar fermented (21,54). The advantage of this type of transport lies in the conservation of ATP (54).

In Spirochaeta aurantia, a facultative anaerobe, only mannitol is transported by a PTS. The product of this reaction is mannitol-1-phosphate. Three proteins are required for this reaction. Two are soluble and on the basis of physiochemical properties are analogous to HPr and EI. The third protein is a membrane-bound EII (61). The regulation of this system differs from the model systems. In these systems HPr and EI are regulated on one operon, whereas the sugar specific components are coordinately regulated as a separate operon. These operons map at distinct loci on the E. coli chromosome (55). In S. aurantia, the three PTS proteins are genetically regulated in a coordinate fashion. In addition, the enzyme responsible for the first

step in mannitol dissimilation, mannitol-1-phosphate dehydrogenase, is also regulated in the operon (55).

Phosphotransferase systems and the regulation of transport. In addition to a transport function, the phosphotransferase systems (PT-systems) in the Gram negative enterics function in the control of non-PTS transport. This control is bilateral: the permeases are regulated directly through allosteric modulation and indirectly by regulating their synthesis (55).

In order to demonstrate a model of regulation, the control of the glc PT-systems over the lac permease of E. coli will be described as an example. A brief review of the lac operon will aid in explaining this model. The controlling factors in transcription of the lac operon are two-fold: a negative modulator, the repressor, and a positive modulator cyclic adenosine 5'-monophosphate (cAMP).

In the absence of inducer (lactose or one of its analogues) a repressor molecule inhibits the transcription of lac RNA by binding to an operator site on the DNA. This operator site is flanked by the promoter site on one side and the structural genes on the other. In the presence of an inducer, a repressor-inducer complex is formed. The binding of the inducer causes the repressor to undergo an allosteric shift and therefore causes it to lose its affinity for the sequence of nucleotides to which it binds. It is evident that in the absence of intracellular inducer the lac permease cannot be expressed and thus by controlling the transport of inducer molecules, the cell is able to control the synthesis of transport systems. The prevention of inducer entry is termed "inducer exclusion" (60).

RNA polymerase binding and initiation of transcription is dependent on the formation of an "open complex," that is, the conformation of the DNA is altered as to produce localized melting. The effect of this is to allow the polymerase to transcribe the lac genes (45).

The formation of the "open complex" is brought about by a second modulating system composed of two elements: cAMP receptor protein (CRP) plus cAMP. This system is analogous to the repressor-inducer complex except that it exerts a positive control. CRP by itself does not exhibit an affinity to the DNA. However when complexed to the low molecular weight effector molecule, cAMP, its affinity towards the DNA increases by virtue of an allosteric transition. The binding site of this complex is on the lac promotor in the region distal from the operator (45). Thus, it is obvious that controlling cAMP synthesis would control the level of expression of the lac permease, and therefore, lactose uptake. Synthesis of cAMP is accomplished by regulation of the allosteric enzyme, adenylate cyclase.

It has long been observed that growth of E. coli in a combination of lactose and glucose results in a biphasic growth pattern. This diauxic growth is a result of the repression of the expression of the lac genes (60). Not only is the inducer (lactose) excluded from these cells but cAMP levels are low. It is during the lag period preceding the second burst of growth, following glucose exhaustion, that cAMP is synthesized and sufficient quantities of inducer are accumulated to induce the lac operon.

If cells are grown on a permissive growth substrate such as glycerol, where the expression of the lac genes occurs in the absence of inducer

and glucose is added, two effects are observed: (1) there is an immediate cessation of lac gene transcription followed by (2) a resumption of transcription at a repressed level. The immediate severe repression is called transient repression and the second type of repression is termed catabolite repression (38). Both these forms of inhibition occur even though the inducer is present intracellularly. On the other hand, both phenomena reflect the cAMP levels measured. That is, glycerol is a permissive substrate because it allows cAMP to be synthesized, whereas growth in glucose causes an inhibition of adenylate cyclase; thus cAMP levels are low in cells growing on glucose (39,46). The addition of glucose to cells growing on a permissive substrate results in a severe transient decrease in intracellular cAMP followed by a resumption of synthesis of this metabolite but at a lower level than that observed in the absence of glucose (38). The lowering of cAMP levels is due to an inhibition of adenylate cyclase (46), excretion is a function of intracellular levels not of growth substrates (45).

Catabolite repression is a misnomer since the effect does not require metabolism of glucose. Non-metabolizable analogues such as α-MG produces the same effect (45,47). The glucose effects (i.e., inducer exclusion, transient repression, and catabolite repression) are exerted with a wide range of metabolizable sugars. Most of these systems have in common the following: they are inducible and they are transported by non-PT-systems, though their transport may be mediated by an ATP-dependent or a proton motive force-dependent mechanism (45,55).

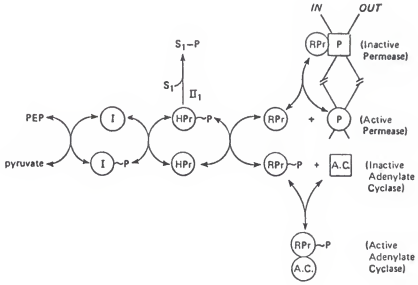
A unifying hypothesis to explain the two underlying mechanisms of repression, inducer exclusion and adenylate cyclase inhibition, has been

proposed by Saier (55). This is diagrammed in Fig. 2. The overall mechanism is a phosphorylation-dephosphorylation modulation of competing functions which are all dependent on a central protein. In the outline, the phosphorylation reaction is mediated by the general PTS proteins, EI and HPr. In the presence of glucose, the equilibrium of the phosphoryl donation lies in the direction of glucose phosphorylation; however in the absence of glucose, the phosphorylated HPr is free to donate the phosphoryl group to a hypothetical protein termed RPr (regulatory protein). In the phosphorylated form, this protein becomes a positive effector for adenylate cyclase, thus allowing cAMP to be synthesized thereby negating catabolite and transient repressions. In the non-phosphorylated form (i.e., when glucose is present), RPr has an affinity for the non-PTS permeases. The proposal is that these permeases are allosteric proteins and that RPr is a negative effector. Therefore, RPr binding inhibits transport of non-PTS sugars and is responsible for inducer exclusion. When phosphorylated, RPr loses its affinity for the permease and the unmodified permeases can then function.

Much evidence has been accumulated to support this model. A selected amount will be summarized here.

Using S. typhimurium and E. coli, it was found that mutations in the genes coding for EI or HPr had a surprising effect on the metabolism of non-PTS sugars such as melibiose and maltose. Tight mutants could not grow on these substrates, whereas the growth pattern of leaky mutants were phenotypically indistinguishable from wild-type. However, if α-MG is added to induced cells, the leaky mutants exhibit a profound increase in sensitivity to the repressive effects of this analogue as

Fig. 2. Proposed scheme for the regulation of carbohydrate transport and metabolism by the phosphoenolpyruvate-dependent phosphotransferase system in E. coli (55). Abbreviations are as follows: PEP, phosphoenolpyruvate; I, Enzyme I: II, Enzyme II; S_1, sugar; S_1-P $sugar_1$-phosphate; RPr, regulatory protein: A.C., adenylate cyclase. The permease refers to a non-PTS permease; i.e., ATP- or proton motive force-dependent transport.


IN
OUT
RPr
P
(Inactive Permease)
S1~P
S1
II1
PEP
I
HPr~P
RPr
+
P
(Active Permease)
pyruvate
I~P
HPr
RPr~P
+
A.C.
(Inactive Adenylate Cyclase)
RPr~P
A.C.
(Active Adenylate Cyclase)

compared to wild-type. This repressive effect is evident in terms of growth and enzyme synthesis (66). The degree of this repression is related to the extent of induction. Thus, fully induced cultures are more refractory towards the repressive effects of α-MG. Also, non-induced cells cannot grow if transferred to a medium containing this glucose analogue and the non-PTS sugar, melibiose (60,63). This indicates that the primary effect being investigated in these studies is inducer exclusion (63). Furthermore, the EII specific for the PTS sugar must be present for the sugar to cause this hypersensitivity (63, 66), substantiating the involvement of PTS-mediated transport.

A second mutation described by Saier and Roseman (62) mapping at a site co-transducible with the pts operon (genes for EI and HPr) but not part of the operon was found to suppress mutations within the pts operon in terms of the hypersensitivity and total repression described above. The distinguishing phenotypic characteristic resulting from these suppressor mutations was found to be depressed levels of the EIII for glucose; i.e., the soluble factor which is part of the high affinity glc PTS. These mutants were termed catabolite repression resistant and the gene responsible was termed crr. It is postulated that this protein ($EIII^{glc}$) may indeed be RPr (60).

A second group of mutants were isolated by Saier et al. (67) which also relieved PTS-mediated repression; i.e., repression due to an EI or HPr mutation. The characteristic that distinguished this second group from the crr mutants, is the specificity of the relief. The crr mutation was general in that both transport and metabolism of all the affected non-PTS sugars were relieved of repression, whereas in this second

category, a number of unique mutants were isolated which exhibited a refractory response when a specific non-PTS sugar was tested. That is, there was a double mutant in which maltose transport and dissimilation was released from repression, one which melibiose transport was released, etc. The mutations responsible for this release mapped in or near the genes coding for the individual transport proteins. These results suggest that an altered permease may be responsible for this relief and thus indicate an allosteric interaction between a PTS component and the permeases (60,67).

Lastly, increased levels of PEP was shown to partially overcome the inhibitory effect of α-MG-induced repression of glycerol transport in wild-type cells. This indicated phosphorylation involving PEP was utilized by these cells to regulate sugar uptake (58).

All these results relate to the transport of non-PTS sugars and thus the regulation of non-PTS-mediated transport. An interaction was also observed between PTS components and adenylate cyclase. However, the effects of the various genetic lesions were not completely analogous. The following observations are relevant to the discussion on adenylate cyclase: (1) Mutations affecting the low affinity EII proteins render the adenylate cyclase of E. coli insensitive to glucose and its analogues (i.e., 2-DG) but not α-MG, whereas mutations resulting in a disfunctional EII of the high affinity system render the enzyme insensitive to α-MG-induced but not 2-DG-induced inhibition (19). These mutations affect the regulation not the activity of the enzymes and these data indicate a functional PTS is required for adenylate cyclase regulation.

(2) In vitro assays of wild-type adenylate cyclase fail to show glucose-mediated inhibition of this enzyme, again pointing to the need for transport activity (47).

(3) EI deletion mutants show low levels of adenylate cyclase activity. Leaky EI mutants are "hypersensitive" to the inhibitory effects of glucose on adenylate cyclase activity and this "hypersensitivity" could be partially overcome in permeabilized cells by the addition of PEP (48,57).

(4) A mutation in the crr gene results in depressed levels of cAMP in cells which have wild-type levels of EI and HPr (48).

(5) Kinetic analysis revealed that adenylate cyclase was still functioning in vivo albeit at depressed levels in the presence of glucose. This tends to indicate positive regulation is required and taken with other data, glucose prevents the positive regulation of adenylate cyclase. These data also suggest that the crr gene product ($EIII^{glc}$) may be involved in regulation. The effect of temperature on the acivity and regulation of adenylate cyclase suggests that a stable complex, i.e., an allosteric interaction, is a prerequisite for activation (48).

The isolation of the crr mutation along with the observation that pre-growth on glucose enhanced the inhibition of transport suggests that a component of the inducible high-affinity glucose system is also involved in inducer exclusion. In support of this, Scholte et al. (73) have shown the identity of the crr gene product to be $EIII^{glc}$ using cross-immunoelectrophoretic techniques.

Other phosphotransferase functions. There is evidence that the PTS functions in the regulation of motility (55). The responsible factor appears to be the sugar-specific EII protein (36). In addition, the EII

protein may play a role in the fermentation of the sugar they are transporting (36). Lastly, the various EII's are able to catalyze the transphosphorylation reaction: the transfer of a phosphoryl group from a derivatized sugar to its non-derivatized counterpart (36,59). The physiological significance of this latter reaction is unknown; however, there has been speculation that it may have a regulatory function (60).

Characterization of the phosphoenolpyruvate-dependent phosphotransferase systems in the strptococci. The literature concerning the streptococci lacks the detail outlined above. As demonstrated in the above discussion, the model system is based on data collected from studies in E. coli and S. aureus. Studies on the streptococci do not involve the molecular dissection of the various PT-systems. Identification of PTS transport in these organisms is based on one or more of the following criteria: stimulation of transport by intracellular PEP reserves and/or PEP-dependent phosphorylation of a sugar, the demonstration that ATP cannot substitute for PEP for either of these two functions, and the identification of the transport product of a sugar and/or its non-metabolizable analogue as a phosphorylated derivative.

The most extensive studies of the lactic streptotocci have been on the lac PTS in Streptococcus lactis. McKay et al. (42) demonstrated that NaF, an inhibitor of enolase which catalyzes the glycolytic step involved in the conversion of 2-phosphoglycerate to phosphoenolpyruvate inhibits the transport of TMG, a lactose analogue. The intracellular product was identified as TMG-P in this and other studies (42,86). Thompson (86) measured PEP utilization along with TMG accumulation and found there was a stoichiometric relationship between these two parameters.

Furthermore, the conditions used in this study did not permit the ATP-dependent accumulation of amino acids; thereby demonstrating that PEP was not being converted to ATP by pyruvate kinase.

In this organism, lactose metabolic enzymes are plasmid coded (32). In a strain devoid of its lac plasmid, both lac PTS and phospho-β-galactosidase activity are absent, suggesting a close linkage between these lac genes. However, regulation of these enzyme activities does not appear to be coordinated. In wild-type, the phospho-β-galactosidase is present under growth conditions which repress the expression of the lac PTS (32). This is an interesting observation in light of other studies on Gram positive bacteria suggesting that the inducer for the lac PT-systems is galactose-6-phosphate. Morse et al. (43) isolated phospho-β-galactosidase negative mutants from S. aureus and found that these cells could not transport lactose when grown under conditions which induce the lac PT-system in wild-type. However, galactose-6-phosphate was able to induce this system in such mutants. As stated in a previous section, incoming lactose is phosphorylated at the C6 of the galactose moiety (78). Also, early reports indicate the constitutive nature of the phospho-β-galactosidase of S. aureus (41).

In strains of S. lactis, galactose is a more potent inducer of the lac PTS than lactose itself (6,32). Galactose has two transport systems in these cells, one which requires ATP and a second which requires PEP. In an elegant series of experiments, Thompson demonstrated the existence of two systems (88). He accomplished this by comparing galactose dissimilation in the presence of glycolytic inhibitors such as iodoacetate or the presence of ionophores. The latter he termed the Gal P system.

He confirmed his findings by using an ATP-generating substrate, arginine, which allowed galactose transport but not PEP-mediated lactose transport. Through competition studies, it was shown that PEP-dependent galactose transport was mediated by the lac PT-system. This is in agreement with LeBlanc et al. (32) who were able to demonstrate the coincident disappearance of the lac and gal PT-systems upon curing a strain of S. lactis of one of its plasmids. In another study, Cords and McKay (6) isolated a revertant of a lac PTS^- strain. However, galactose could not induce the lac-PTS in these cells and upon closer examination, it was shown that these cells only possessed the gal P system.

Transport via these two different systems results in the dissimilation of galactose by two different routes. If galactose is translocated by the gal P system, it enters the cell cytosol as a free sugar. Subsequently, it is phosphorylated by galactokinase and ATP (6), and is metabolized via the Leloir pathway (88) to glucose-1-phosphate and consequently by the Embden-Myerhof pathway upon isomerization to glucose-6-phosphate (90). Galactose-6-phosphate is the product of PTS transport. This compound is metabolized through the tagatose pathway (43,88). This biochemical sequence involves the conversion of galactose-6-phosphate to tagatose-6-phosphate which is phosphorylated by ATP to tagatose-1,6-diphosphate and finally to dihydroxyacetone phosphate which can feed into the Embden-Myerhof path (87). It should be noted that in these cells, lactose is phosphorylated at the C6 of the galactose moiety. Thus it also must be metabolized via the tagatose pathway with the free glucose produced by the action of phospho-ß-galactosidase being processed through the Embden-Myerhof pathway (87).

There appears to be some controversy as to which galactose transport system is the predominant mode of transport. Based on their biochemical analysis of mutants, Cords and McKay (6) speculated that galactose-6-phosphate repressed the gal P system. However, Thompson (88) found that the gal P had a 10-fold lower K_m for galactose than the lac PTS. In addition, he found growing cells contained low levels of tagatose phosphate but high levels of fructose phosphate which indicated the Leloir path was operating. He interpreted these results to mean that the Leloir path is the predominant route of galactose degradation in relatively low levels of sugar and thus represents the major route.

In addition to these two types of galactose transport systems, a second gal PTS appears to exist (44,88). This system is specific for galactose and thus gal^+ lac^- cells can only grow on galactose. Other phenotypic traits of this strain are the inability to accumulate TMG and the production of galactose-1-phosphate and galactose-6-phosphate. As already discussed, these are the first intermediates in galactose metabolism when galactose is transported by the gal P and the gal PTS, respectively. Thus, these cells which are lac PTS deficient are still able to transport galactose through a PTS in addition to a proton-driven permease. It is apparent that galactose transport in this organism is complicated.

A second PEP-mediated transport system in S. lactis has been defined for glucose. Thompson (86) has shown that there is a stoichiometric relationship between PEP depletion and 2-DG uptake. He also demonstrated that the selective use of inhibitors of glycolysis such as P-chloromecuribenzoate (target: glyceraldehyde-3-phosphate dehydrogenase) leads to

inhibition of 2-DG uptake in metabolizing cells. Based on the following criteria this glc PTS was classified as being similar to the low affinity E. coli system: (1) it was refractory to the inhibitory effects of sulfhydryl reagents, (2) the following glucose analogues appeared to compete with ^{14}C-glucose: 2-DG, mannose, and N-acetyl-glucosamine, while the substrate of the high affinity system, α-MG, was without effect, and (3) it is a constitutive system (86).

It appears that the predominant transport system for sugars in the cariogenic streptococcus, S. mutans is of the PEP-dependent phospho-transferase type. This is not surprising in view of the fact that this is an aerotolerant anaerobic organism (85), and, therefore, must conserve its energy reserves. The PT-systems studied to date in this organism are outlined in Table 1. The literature contains an extensive survey of the various transport systems found in the species; however, details of the individual systems are lacking.

The first system to be defined was the glc PTS. Schachtele and Mayo (71) demonstrated a PEP-dependent phosphorylation reaction involving 2-DG in permeabilized cells of S. mutans. ATP could not replace PEP in this reaction. Supporting evidence for a PEP dependent transport system came from the observation that NaF inhibited the uptake of 2-DG. Based on the failure of 6-DG to inhibit phosphorylation of 2-DG, they concluded that this analogue is phosphorylated at C6. These investigators extrapolated their findings to the natural substrate, glucose. Even though it is an obvious assumption that 2-DG is transported and phosphorylated by the same proteins involved in glucose uptake, competitive inhibition studies to prove this point have never been done.

Table 1. Phosphoenolpyruvate-dependent phosphotransferase systems in S. mutans.

Glucose (Schachtele and Mayo, 1973)
Mannitol (Maryanski and Wittenberger, 1975)
Sorbitol (Maryanski and Wittenberger, 1975)
Lactose (Calmes, 1978)
Sucrose (St. Martin and Wittenberger, 1979)

Two other monosaccharides studied in this organism are the hexitols: sorbitol and mannitol. Both of these sugars are substrates of a PEP-dependent phosphorylation reaction. These PT-systems are inducible and therefore are evident in cells only when grown on the respective sugars. The enzymes, mannitol-1-phosphate dehydrogenase and sorbitol-6-phosphate dehydrogenase are coinduced indicating a regulon codes for these PT-systems (40).

The most thoroughly investigated PTS in this organism is the sucrose (suc)PTS. The reason for this is the medical implication of sucrose metabolism in this species. This organism is able to synthesize a dextran capsule through the action of a glucosyltransferase and it is this capsule that allows adhesion to tooth surfaces (13,14).

Slee and Tanzer (80,81) described a unique PTS for sucrose in this species. Using a variety of strains, they demonstrated a PEP-dependent phosphorylation of sucrose to sucrose-6-phosphate which appeared inducible; i.e., it was not present in glucose adapted cells. St. Martin and Wittenberger (83,84) verified the inducibility of the suc PTS by employing a greater variety of growth substrates.

Upon translocation of sucrose, the dissacharide is phosphorylated at the C6 position of the glucose moiety (83,84). Hydrolysis of sucrose-6-phosphate requires a unique invertase. A sucrose-6-phosphate hydrolase was described in S. mutans (5,84). This enzyme had a high affinity for its substrate and thus could only be detected directly by using sucrose-6-phosphate. It is synthesized constitutively, unlike the suc PTS (84). It is interesting to compare this system to the lactose system in S. aureus. In this system galactose-6-phosphate is the inducer (43) and this compound

is the product of a constitutive hydrolase (41). It may be that sucrose-6-phosphate hydrolase activity results in the production of the suc PTS inducer. Indeed, sucrose-6-phosphate hydrolase negative cells which have an evident suc PTS are isolated as PTS constitutive (84). However, using the S. aureus analogy, it seems unlikely that glucose-6-phosphate is the inducer since glucose-grown cells have repressed levels of suc PTS activity (81,83,84). The other product resulting from the action of this hydrolase is fructose. Growth of cells on fructose results in lower levels of the suc PTS than growth on glucose (83). Further experiments must be performed to clarify these contradictory data.

Thompson and Chassy (89) have recently described a suc PTS in S. lactis. It is interesting that in this organism they found the sucrose-6-phosphate hydrolase to be inducible. They also found a fructokinase which was induced in sucrose-grown cells. The identification of this latter enzyme in cells metabolizing sucrose along with the finding that the cytosol of these cells contains high levels of free fructose, lead these investigators to conclude that the fate of the fructose resulting from the hydrolysis of sucrose-6-phosphate was phosphorylation by an ATP-dependent kinase.

Recently a suc PTS has been reported in E. coli (35,72). This is an interesting finding since it had been previously thought that in this organism the PTS was used exclusively for the transport of monosaccharides (55). The ability to utilize sucrose is a plasmid-borne function, the origin of which appears to be Klebsiella pneumoniae (35). This plasmid bears two genes coding for sucrose metabolic enzymes, EII^{suc} and sucrose-6-phosphate hydrolase (72). There are some interesting features of this

system. First, there is a coordinate induction of these two proteins and genetic studies suggest that the inducer is fructose or its phosphorylated derivative. This is different from S. mutans where fructose appears to be a repressive sugar for the suc PTS (84). In addition the genes for these two proteins do not appear to be transcribed as a regulon in S. mutans. Additional evidence that a regulon exists in E. coli is the isolation of a mutant constitutive for both proteins. Another unusual feature is the apparent requirement for the $EIII^{glc}$. This was demonstrated by transducing the suc PTS into EIII negative strains of E. coli and was confirmed using in vitro reconstitution of the individual protein (35).

Using the criteria of PEP dependency, NaF inhibition, and product isolation, Calmes (3) concluded that lactose uptake in S. mutans is mediated by a group translocation mechanism. He used a variety of assays to study this system including the hydrolysis by permeabilized cells of the lactose analogue, o-nitrophenyl-β-galactopyranoside (ONPG), in the presence of PEP and the transport of TMG into intact cells followed by the extraction and identification of its phosphorylated derivative.

A phospho-β- galactosidase has also been identified (16,17) and characterized (4) in this species. Conflicting data as to the regulation of this enzyme have been reported. Results published by Calmes and Brown (4) suggest that this is an inducible enzyme. Furthermore, the preferred inducer is lactose. Galactose is also an inducer but the amount of the enzyme synthesized in the presence of this monosaccharide is less than that obtained upon lactose induction.

These results parallel those obtained by Calmes (3) when investigating the lac PTS; namely, both lactose and galactose are inducers with the dissacharide more active in this regard. These findings are in direct disagreement with the induction pattern of the lac PTS postulated to occur in S. aureus. The results of Hamilton and Lo (17), however, suggest a similarity between the mechanisms of induction of the lac PTS in S. mutans with that in S. aureus. That is, in S. mutans the levels of phospho-β-galactosidase vary to some extent with the growth condition; however, this enzyme is always present. Also the levels are highest when cells are grown in galactose even though with some strains tested the relative levels were close. The reasons for the discrepancies between the authors may be due to the different strains used in the respective studies. Finally, in a later report published by Hamilton and Lebtag (16), much more dramatic increase in the levels of phospho-β- galactosidase are shown in cells grown in lactose and galactose when compared with glucose. For both the lac PTS and phospho-β-galactosidase, galactose is a better inducer than lactose. They interpret their data to mean a co-regulation of the lac PTS and phospho-β- galactosidase genes.

Enzymes of the tagatose pathway have been demonstrated in S. mutans (16). The levels of these enzymes increase, though not as dramatically in all strains, when lactose is the carbon source. Furthermore, this increase is evident only when lactose, not galactose, is included in the growth medium. Growth in galactose induces enzymes of the Leloir pathway (16). Thus in this respect, lactose metabolism follows the model defined in S. lactis.

Most of the work on transport in the streptococci has been physiological in nature. The biochemistry of PT-systems in these organisms has not been extensively investigated. One exception is the study of the glc PTS of S. faecalis. Interestingly, the PTS appears to be similar to that of the low affinity of E. coli in that the EIII has been characterized to be membrane bound and complexed to EII (27).

Two attempts at dissecting the PTS in S. mutans are noteworthy. Schachtele (70) isolated membranes from glucose-grown cells of S. mutans. These membranes alone were able to carry out a PEP-dependent phosphorylation of glucose. Since the method of membrane preparation was crude and yielded impure membranes, definitive conclusions as to the cellular location of the various components could not be drawn. Another study by Maryanski and Wittenberger (40) led to equally unsatisfactory results. They investigated the localization of components of the glc and mannitol (mtl) PT-systems by recombining soluble and particulate extracts of induced and non-induced cells and determining the amount of sugar phosphorylation. In recombining extracts of glucose-grown cells, they were able to demonstrate the phosphorylation of glucose but not the phosphorylation of mannitol . Soluble and particulate fraction from mannitol-induced cells recombined to phosphorylate both mannitol and glucose (i.e., glucose is constitutive). More importantly, they were able to demonstrate a mannitol reaction when the glucose soluble and mannitol particulate fraction were recombined. Alone neither one catalyzed phosphorylation of mannitol. This indicated that both soluble and particulate components exist in S. mutans. However, in both cases the soluble components alone were as active in phosphorylating activities, indicating that the method

of membrane preparation (sonication) resulted in the formation of small membrane fragments. Interestingly, in constrast to Schachtele's work, the pellets alone did not show activity, supporting the conclusion that extreme fragmentation of membranes occurred during sonication. This apparent failure to clearly separate the particulate and soluble fraaction make any interpretation difficult.

Maryanski and Wittenberger (40) were able to demonstrate the presence of a heat stable component that could enhance phosphorylation of mannitol by cell-free extracts. One minor but perhaps significant point is the ability of Ca^{2+} to replace Mg^{2+} in their assay system and the inhibition that Zn^{2+} produced. In E. coli these ions had opposite effects (30). Mg^{2+} is required for the EI catalyzed transfer (25); therefore, this difference may reflect a subtle diversity between EI components.

Regulation of carbohydrate utilization in Gram positive organisms

The systems of regulation in the Gram negative enterics have been studied extensively by a number of laboratories. Much of this work has been concerned with Saier's model (55) of regulation. To date very little is known about regulation of transport in Gram positive cells and much less is known about the streptococci. One reason for this lack of knowledge may be the absence of genetic systems of study in these organisms.

As with studies of sugar transport, studies of regulation by these organisms have progressed little beyond the descriptive stage. Hamilton and Lo(17) surveyed various oral streptococci for the induction of lactose metabolism. A comparative study of S. salivarius and S. mutans was performed. Their data did suggest that the predominant lactose pathway in S. salvarius was via β-galactosidase indicating the presence

of a non-PTS permease, whereas S. mutans contained phospho-β-galactosidase indicating a dependence on the PTS for transport. They based their conclusions on the observation that ONPG could be hydrolyzed by permeabilized cells of S. salivarius with equal efficiency in the presence or absence of PEP. However, this result could be due to a large energy reserve within these cells. Interestingly, they observed a true diauxie with S. salivarius when these cells were grown in equimolar lactose and glucose, where in S. mutans lac PTS induction under these conditions was observed at low glucose concentrations. In a parallel experiment they demonstrated that isopropyl-β-D-thiogalactopyranoside (IPTG) and galactose could induce lactose metabolic enzymes in glucose-grown cells of both S. mutans and S. salivarius. The obvious contradiction of these two experiments is difficult to reconcile. The latter experiment employed 28 mM glucose as a growth substrate; however, the authors do not mention the amount of glucose remaining at the time of the addition of 8 mM IPTG. The addition of IPTG alone (control) to cells of S. salivarius produced a greater amount of β-galactosidase activity than when glucose was present, indicating a repressive effect of glucose; however, the authors made no mention as to the growth substrate in these control cells. The analogous data obtained for S. mutans were not presented.

A diauxic growth pattern occurs when glucose-grown cells are transferrred to a medium containing equimolar glucose and sucrose (82). The second phase of growth correlates with induction of the suc PTS.

The mechanism (s) which allows preferential sugar utilization in Gram positive organisms has not been elucidated. The model evoked to explain this form of regulation in Gram negative cells is inadequate. One reason for the non-applicability of Saier model is the failure to

define a role for cAMP in these organisms. In studies on Bacillus subtilis (8), Lactobacillus plantarum (20,68), Bacillus megaterium (91), and S. faecalis (24), cAMP addition could not relieve repression. Interestingly, in two separate studies, S. mutans was found to respond to cAMP and adenylate cyclase was detected (17,51). However, in one case the methodology used to detect the enzyme has been shown to give artifactual results (45). It is more difficult to evaluate the second study in which the reliable method of radioimmunoassay was used to detect cAMP since no subsequent reports have appeared in the literature. The second reason it is difficult to apply the Saier model to many Gram positive organisms is that in these bacteria we see one PTS sugar (glucose) regulating other PT-systems (usually for dissacharides) rather than PTS regulation over non-PTS transport.

In order to explain the hierarchy of sugar utilization via the various PT-systems, Thompson et al. (90) evoked the concept of catabolite inhibition. This mechanism involves the differential affinities of the various sugar-specific components for HPr~P. For example in the above discussion concerning S. mutans, the sucrose-specific components would have a lower affinity for the HPr~P than the glucose-specific moieties.

Catabolite inhibition could explain the diauxic effect observed in non-induced cells. For instance, in S. faecalis, the kinetics of inhibition by glucose of the lac PTS appear to be competitive (23). Alternatively, this concept may be applied to the inhibition observed with pre-induced cells. For example, glucose inhibits the uptake of galactose (PTS-mediated) in galactose-grown cells of S. lactis (90). In the former case, catabolite inhibition would result in a situation analogous to

inducer exclusion (23), whereas a general repression would be observed in the latter case (90).

Another theory put forward involved inhibition by sugar-phosphates (60). It is postulated that a sugar phosphate binding site of the EII may be a means of turning off transport. For instance, an increase in intracellular glucose-6-phosphate was shown to be coincident with a decrease in fructose (frc) PTS-mediated transport in E. coli. Saier and Simoni (65) have shown that lactose uptake in S. aureus inhibits uptake of other PTS sugar. These depressed rates of uptake are dependent on the lactose-specific PTS components being functional. They suggest that galactose-6-phosphate is responsible since a lag precedes inhibition when the cells are presented with two sugar substrates (i.e., lactose phosphate must be processed to produce the putative inhibition). On the other hand, Thompson et al. (90) rules out inhibition by sugar-phosphates as being the cause of glucose inhibition of galactose uptake in pre-induced cells of S. lactis since the levels of glucose-6-phosphate were essentially invariable in cells growing on galactose compared to glucose. The little-understood transphosphorylation reaction has been interpreted by Saier and Moczydlowski (60) to support their proposal of sugar phosphate mediated-regulation; however, in transphosphorylation the binding site on EII is only for a sugar phosphate derivative homologous to the underivatized form.

A third type of proposed regulation is relevant here especially when considered with the discussion to follow; this involves the inhibition of α-MG uptake by an energized membrane. The inhibition can be reversed by the use of ionophores and other inhibitiors of the proton motive force (60).

In addition to the regulation observed when cells are presented with two different growth substrates, there is a regulatory mechanism imposed by growth. A secondary transport system for glucose exists in S. mutans. This is apparently dependent on an energy source other than PEP and evidence acquired by the use of inhibitors such as the ionophore carbonyl-m-chlorophenylhydrazone suggest it is mediated by a proton pump. The affinity of this system for glucose is 8-15 times lower than the PTS (19). Thus at lower glucose concentrations, the glc PTS would be expected to predominate. This prediction was shown experimentally using the controlled conditions of a chemostat: cells grown under a low dilution rate utilized the PTS (11,18). Surprisingly, glycolytic activity was found to be greater under conditions of glucose starvation than when excess glucose was present. At the higher dilution rate (excess glucose) the PTS was apparently repressed and the low affinity system predominated. The degree of repression was found to be proportional to the growth rate (11).

As with the glc PTS, the suc PTS of S. mutans appears to be inhibited by rapid growth and excess substrate. This has been demonstrated both in batch (82) and chemostat grown culture (10). In the latter study, the investigators calculated that the amount of transport was insufficient to account for the total sucrose uptake observed. Rapidly growing wild-type cells in batch culture had a lower specific activity for the suc PTS than did stationary-phase cells. Mutants were able to grow in sucrose even though they were apparently devoid of suc PTS activity. The authors in both studies interpreted these data to indicate the existence of a secondary transport system. The major problem with interpretation of

the results of the latter study is that the extent of leakiness of these mutants was not adequately quantitated. No activity was detected using an enzyme-linked assay which indicated that this mutation was tight; however, growth on sucrose was approximately 2-log below wild-type. PTS activity below the level of detection of the assay could account for the sucrose-supported growth of the mutants. Further studies are needed in order to determine definitively the existence of an alternate system.

Relevant to these observations is the inhibition of α-MG uptake (PTS-mediated) in E. coli observed during rapid growth. This inhibition may be related to the energized state of the membrane (60). Interestingly, repression of the glc (11) and suc (82) PT-systems in S. mutans is pH dependent; however, activity is increased at the lower pH. If a pH potential imposed by an ATPase is responsible for regulation, activity of the ATPase would result in activation of these PT-systems according to these results since the action of the ATPase cause a decrease in the external pH.

Regulation of sugar transport in the streptococci has been largely speculative; documentation of such regulation has been sparse. The major problem is the lack of biochemical information concerning the various PT-systems. It is not feasible to propose a regulatory protein such as the $EIII^{glc}$ if it is not known whether the glucose system contains an EIII-type molecule. Until now, none of the individual PTS components has been shown to exist in S. mutans. Furthermore, mutants which totally lack a functional glc PTS have not been obtained for this species. Hamilton and St. Martin (18) isolated a glc PTS-mutant from S. mutans which retained 15% of the activity of wild-type. It is difficult to

attribute a regulatory function to the glc PTS using such a leaky mutant.

The first step in understanding regulation of sugar transport is a more comprehensive assignment of PTS components. This work attempts to elucidate, in part, the biochemistry of the S. mutans glc PTS and to relate this to the physiological regulations observed in this organism.

MATERIALS AND METHODS

Cultures and cell growth. Cells of Streptococcus mutans GS5 were routinely maintained in a tryptone-yeast extract (TYE) broth (17). This medium was composed of 10 g/l tryptone (Difco Laboratories, Detroit, MI), 5 g/l yeast extract (Difco Laboratories) and 3 g/l dibasic potassium phosphate. For maintenance, this medium was supplemented with 20 mM glucose (Sigma Chemical Co., St. Louis, MO).

For assay purposes, the cells were grown overnight in TYE broth plus 20 mM of a given sugar. These cells were transferred to a defined medium (DM) broth designed for S. mutans (85). To this defined medium, 5 mM sugar was added. A 10% inoculum size was routinely used. Unless otherwise specified, cells were allowed to grow until late-log/early-stationary phase in the DM; after which time, they were harvested by centrifugation at 12,000 x g for 10 min and washed once in 100 mM sodium phosphate (PB), pH 7.0, plus 5 mM $MgCl_2$. The centrifuge used for routine purposes was a Sorvall RC-5B (Dupont Instruments, Newtown, CN).

Cell preparation for assays. In all the assays outlined below, decryptified cells were used. The decryptification conditions were as follows: washed cells were resuspended in PB, pH 7.0, plus 5 mM $MgCl_2$ to one-tenth their original volume. A toluene-acetone mixture (1:3 v/v) was used to permeabilize the cells. The mixture was added in a ratio of 100 µl/ml of cell suspension and decryptification was carried out by vigorously shaking on a Vortex Mixer (Scientific Industries, Bohemia, NY) for 1-2 min intervals interspersed with cooling in ice. The total length

of shaking was 3-5 min. This methodology was based on that used by Calmes (3) for S. mutans. If a further dilution was required 100 mM PB plus 5 mM $MgCl_2$ was added after decryptification to give the desired cell concentration.

Determination of the lactose phosphotransferase system and phospho-β-galactosidase. The assay for the determination of the lac PTS utilized the lactose analogue o-nitrophenyl-β-galactoside (ONPG; Sigma Chemical Co). The standard reaction mixture contained 100 mM PB, pH 7.0, 5 mM $MgCl_2$, 8 mM sodium phosphoenolpyruvate (PEP; Sigma Chemical Co.), 8 mM ONPG, and cells (concentrations to be detailed in legends to Tables and Figures). The total volume was 0.6 ml. This was then incubated for 30 min at 37 C after which time the reaction was stopped by the addition of 1 ml of a 5% Na_2CO_3 solution (aqueous). The cells were centrifuged in a Sorvall table top centrifuge and the amount of o-nitrophenol (ONP) formed was determined by reading the absorbancy of the supernatant at 420 nm in a Gilford 2600 recording spectrophotometer (Gilford Instruments, Inc., Oberlin, OH). The amount of ONP was then determined from a standard curve and the results are expressed in terms of μmoles of ONP/μg cells (dry weight).

For the determination of phospho-β-galactosidase activity, the lactose-phosphate analogue, o-nitrophenyl-β-galactose-6-phosphate (ONPG-6-P; Sigma Chemical Co.) was used. Unless otherwise specified, the reaction mixture contained 100 mM PB, pH 7.0, 5 mM $MgCl_2$, 10 mM ONPG-6-P, plus cells (amounts to be detailed in legends) in a total volume of 0.5 ml. After 30 min at 37C the reaction was terminated with 5% Na_2CO_3, the cells were removed by centrifugation, and the absorbancy of the supernatant was determined at 420 nm. The results were expressed as the μmoles ONP formed/

µg of cells (dry weight). The assay for the lac PTS was based on that described by Calmes (3) and that for the phospho-β-galactosidase was described by Calmes and Brown (4).

Phosphotransferase assay: LDH/NADH-linked. For general surveys, an LDH/NADH-linked assay was used (29). A mixture containing 80 mM PB, pH 7.0, 4 mM $MgCl_2$, 10 mM PEP, .025 mg lactate dehydrogenase (LDH; Sigma Chemical Co.), 3 mM β-nicotinamide adenine dinucleotide, reduced form, disodium salt (NADH; Sigma Chemical Co.) plus cells (amounts given in legends to Tables and Figures) was monitored at 340 nm in a Gilford 2600 recording spectrophotometer for NADH oxidase. An initial rate was obtained by allowing the reaction to proceed for 4-5 min. The PTS reaction was initiated by the addition of 1 mM sugar substrate. The final volume was 1.0 ml. The reaction was monitored by measuring the decrease in absorbancy at 340 nm for 4-5 min. An initial rate was obtained from this measurement and corrected for the endogeneous NADH oxidase activity. The µmoles of NADH remaining was determined from a standard curve and from this value the amount of NAD^+ formed was determined. Results are expressed as µmoles NAD^+ formed/µg cells/min.

Phosphotransferase assay: radioactive. For a more quantitative analysis, a radiolabelled PTS substrate was used. A typical reaction mixture contained 30 mM PB, pH 7.0, 1.5 mM $MgCl_2$, 10 mM PEP, 10 mM sodium flouride (NaF, Sigma Chemical Co.), and 0.1 mM unlabelled sugar to which was added the specific isotope (New England Nuclear, Boston, MA). The amount of each isotope contained in each reaction mixture is given under the legends to the Figures or in the Results section. To standardize the reaction, 90-110 µg of cells was used and the concentration was determined from a standard curve of dry weight vs. absorbancy of cells at 600 nm

(to be described in a following section). For routine purposes, the time of incubation was 30 min; for kinetic studies, the time was 10 min, since the extent of the reaction was linear within this time. Unless otherwise stated, these studies were carried out at 25 C.

To assay cell-free membranes the following modifications were made: the buffer concentration was generally increased to 80 mM, PB, pH 7.0, the $MgCl_2$ concentration was increased to 4.0 mM and the membrane concentration varied as specified in the RESULTS. Incubation was always at 37 C. In subsequent assays, the volume was scaled down to 200 µl in order to conserve material and 4 mM 2-mercaptoethanol was added.

These reactions were stopped by diluting 100-200 µl of the reaction mixture 1:9 (v/v) in 1% sugar or when the scaled down assay was used, by adding cold 1% sugar in excess of the reaction volume directly to the assay mixture. In both cases, these mixtures were rapidly cooled. From this, the phosphorylated derivative was separated from the labelled substrate by filtration under vacuum through a DE-81 anion exchange filter (Whatman, Clifton, NJ) which had been prewashed with a 1% solution of underivatized sugar. The filters were washed one time with 1% sugar and 4 x with cold H_2O. They were then counted in a Beckman LS8000 scintillation counter (Beckman Instruments, Inc., Fullerton, CA). This assay is based on the methodology developed by Simoni et al. (76). The scintillation fluor used was Aquasol (5 ml; New England Nuclear).

Transphosphorylation. In order to assay directly for the EII^{glc}, the glucose-glucose-6-phosphate exchange reaction was employed. The procedure was a modification of that developed by Saier (59). In this procedure, 70 mM PB, pH 6.0, 3.5 mM $MgCl_2$, 3.5 mM 2-mercaptoethanol,

50 mM glucose-6-phosphate (Sigma Chemical Co.), 10 mM NaF and 50 μM D-[^{14}C(U)]-glucose (4 μCi/μmole) were reacted with membranes. The total reaction mixture volume was 200 μl. The mixture minus the labelled glucose was prewarmed to 37 C and the reaction was begun by the addition of D-[^{14}C(U)]-glucose. Incubation continued at this temperature for 30 min after which time the reaction was stopped by the rapid addition of cold H_2O and the separation of derivatized product from reactant was accomplished by passing the mixture through a Dowex column, AG1-X8 (Cl^-), (0.7 x 4 cm; Biorad Laboratories, Richmond, CA). The column was washed with approximately 3 column volumes of H_2O and glucose-6-phosphate was eluted in 5 ml of 1 M $LiCl_2$ directly into a scintillation vial. To this vial, 10 ml of Aquasol was added and counts were obtained in a Beckman LS8000 liquid scintillation counter. Cpm were converted to dpm by comparison to a standard quench curve (external standard vs. percent efficiency). Results are expressed as μmoles glucose-6-phosphate formed/μg cells (dry weight).

Assay for Enzyme I. To detect this enzyme, the technique of Saier et al. (64) was used. The reaction mixture was composed of 40 mM tris-(hydroxymethyl)-aminomethane hydrochloride (Tris-HCl), pH 7.5, 8 mM $MgCl_2$, 10 mM NaF, 2 mM sodium pyruvate, 0.2 mM phosphoenol[1-^{14}C]pyruvic acid, cyclohexylammonium salt (10 μCi/μmole) plus cell extract. The total reaction volume was 100 μl. All components except the radioactive substrate were incubated at 37 C and the reaction was begun by the addition of the substrate. Incubation continued for an additional 60 min after which time 0.4 ml Sigma color reagent (Stock no. 505-2; 20 mg/100 ml of 2,4-dinitrophenylhydrazine in 1 N HCl) was added. The tubes were mixed thoroughly and incubated for an additional 10 min

at 37 C. The derivatized radioactive product was separated by the addition of 1 ml ethyl acetate. After vigorous mixing, a 600 µl sample was removed from the organic phase, placed in 5 ml Aquasol, and counted in a Beckman LS8000 liquid scintillation counter. Results are calculated per 600 µl product counted and are expressed as pmoles ^{14}C-pyruvate formed/µg protein.

Growth curves. Cells were pre-grown in DM supplemented with 5 mM sugar. At log-phase, the cells were transferred to fresh medium supplemented with sugar(s) at a concentration of 5 mM/sugar. Growth was monitored with a Klett-Summerson photoelectric colorimeter using a red filter #66 (Klett Mfg. Co., Inc., New York, NY) and/or a Gilford 2600 spectrophotometer (600 nm).

Sugar determination in spent medium. Lactose was determined by the Boehringer-Mannheim lactose/galactose kit (Boehringer-Mannheim Biochemicals, Indianapolis, IN). The basic principle of this kit is to convert the lactose to galactose plus glucose by β-galactosidase. The galactose is oxidized to galactonic acid by galactose dehydrogenase plus NAD^+. The appearance of NADH is detected at 365 nm. A 100 µl aliquot of the spent medium was incubated with NAD^+ plus 1.2 U of β-galactosidase for 10 min at 25 C. After this incubation, PB, pH 6.8, and H_2O was added to bring the volume to 1.34 ml. The solutions were first read at 365 nm to obtain a background and the reaction was then started by the addition of 0.4 U of galactose dehydrogenase. Incubation proceeded for 15 min. (The reaction was determined to be complete at the end of this time period.) The final volume of the reaction mixture was 1.36 ml, the NAD^+ concentration was 0.45 M and the PB concentration was 0.15 M. Controls

lacking β-galactosidase demonstrated the absence of galactose from the medium as a contaminant or as an excreted end-product. The concentration of lactose was determined by comparison to the reduction of NAD^+ by a known quantity of lactose.

Fructose was determined by the Boehringer-Mannheim glucose/fructose kit. This kit contains hexokinase plus adenosine 5'-triphosphate (ATP) to convert fructose to fructose-6-phosphate, phosphoglucose isomerase to convert fructose-6-phosphate to glucose-6-phosphate, and glucose-6-phosphate dehydrogenase plus β-nicotinamide adenine dinucleotide phosphate ($NADP^+$) to convert glucose-6-phosphate to gluconate-6-phosphate plus NADPH (reduced). The results were read at 365 nm and the amount of fructose calculated from a standard. Controls lacking phosphoglucose isomerase demonstrated the lack of glucose in the samples tested. The procedure involved incubating 100 µl of the spent medium with 1.0 M $NADP^+$, 4.0 M ATP, 1.33 U hexokinase, and 0.67 U glucose-6-phosphate dehydrogenase. The reaction mixture was incubated for 15 min at 25 C after which time 3.27 U of phosphoglucose isomerase was added to all but the controls and incubation was continued for an additional 15 min. The results were obtained by reading the assay at 365 nm in a Gilford 2600 spectrophotometer.

The glucose determination was based on the glucose oxidase method of Raabo and Terkildsen (50). All reagents were from a diagnostic kit (Sigma Chemical Co.). Briefly, glucose is oxidized to gluconic acid plus H_2O_2. The presence of H_2O_2 is detected by its reaction with o-dianisidine which, when oxidized, becomes brown and can be detected at 450 nm. Typical samples (0.1 ml) contained up to 15 µg of glucose.

To this 1.0 ml of the reagent was added. This reagent contains 5 U/ml glucose oxidase, 1 Purpurogallin U/ml of peroxidase, and 4 µg/ml of o-dianisidine. After mixing, the reactants were incubated at 37 C in the dark for 30 min.

Membrane preparation. The procedure used is a modification of the one developed by Siegel et al (75). Typically, cells were grown in 200 ml of either DM or TYE plus 20 mM glucose. At the log-phase, the cells were harvested and washed 2 x in 0.9% NaCl, 2 x in 5 mM ethylenediaminetetraacetic acid(EDTA), and 2 x in 20 mM Tris-HCl, pH 6.8, plus 5 mM 2-mercaptoethanol and 10 mM $MgCl_2$("lysis buffer"). The cells were frozen at some point during the washing procedure. For lysis, cells were suspended in 50 ml lysis buffer with 5 mg purified mutanolysin (Ml; a gift from Kanae Yokagawa, Dainippon Chemical Co., Tokyo, Japan) and incubated 60 min at 37 C; after which time, 2.5 mg RNase and 2.5 mg DNase (Sigma Chemical Co.) were added and incubation continued with stirring for an additional 60 min at 37 C. Since it was determined that the DNase preparation contained proteolytic activity, phenylmethylsulfonyl fluoride (Sigma Chemical Co.) was included in this last step. Membranes were collected by centrifugation at 30,900 x g for 60 min and washed once in 100 mM PB, pH 7.0, containing 5 mM $MgCl_2$ and 5 mM 2-mercaptoethanol. The supernatant from the first and second centrifugation were pooled, dialyzed for 48 h against H_2O (with at least 3 changes), lyophylized, and resuspended in 25 ml of 100 mM PB, pH 7.0, containing 5 mM $MgCl_2$ and 5 mM 2-mercaptoethanol. (This will be referred to as the cytoplasmic fraction.) The cytoplasmic fraction was stored at -4 C. The pellet was resuspended in the same buffer and subjected to a low

speed spin at 1075 x g for 5 min in order to remove whole cells. The supernatant from this step was then centrifuged at 30,900 x g for 60 min to recover the membranes in the pellet. The purified, cell-free membranes were resuspended in 2.5 ml of the above buffer and stored at -4 C. These were free from contamination by cell wall (75).

Cell wall-membrane complexes were prepared using the method of Bleiweis et al. (2). For this procedure, cells were grown to early-stationary phase in 200 ml DM supplemented with 20 mM glucose. They were harvested and washed twice with 100 mM PB, pH 7.0, containing 5 mM $MgCl_2$. The washed cells were then resuspended in 10 ml of this buffer plus an approximately equal volume of glass beads and 100-200 µl of tributyl phosphate, and homogenized for 3 min in a Braun tissue homogenizer (Bronwill Scientific, Rochester, NY) cooled by CO_2. The beads were removed by filtration through a scintered glass filter (coarse). During this process, the beads were washed with several volumes of buffer. The final suspension volume was 200 ml. DNase, 10 mg, and RNase, 10 mg, were added and enzymatic digestion was allowed to proceed for 2 h at 37 C under conditions of constant stirring. EDTA, 3 mM, was added and the membranes were pelleted out and washed 1 x in 100 mM PB, pH 7.0, containing 3 mM EDTA, and finally resuspended in 100 mM PB, pH 7.0, containing 5 mM $MgCl_2$. This was then subjected to a 1075 x g centrifugation for 5 min and the membranes were collected by centrifuging the supernatant at 30,900 x g for 60 min. The "Braun-membranes" were stored at -4C in 2.5 ml 100 mM PB, pH 7.0, containing 5 mM $MgCl_2$.

A third method of cell breakage involved homogenizing the cells in a Bead Beater (Biospec Products, Bartlesville, OK). This apparatus

is analogous to a Waring Blender except that it requires glass beads. One liter of cells grown in DM plus 20 mM glucose was harvested at early-stationary phase. After harvesting and washing twice, they were resuspended in 100 mM PB, pH 7.0, containing 5 mM $MgCl_2$ and 5 mM 2-mercaptoethanol and homogenized using a 200-300 ml volume of glass beads for 5 min. To prevent foaming, tributyl phosphate was added prior to homogenizing. After filtering through scintered glass (coarse) to remove the glass beads, the suspension was treated with DNase and RNase, 50 µg each, for 2 h at 37 C with constant stirring. The cell wall-membrane complexes were then collected and washed twice in 100 mM PB, pH 7.0, containing 3 mM EDTA; whole cells were removed by centrifugation and the cell wall-membrane complexes were pelleted and resuspended in 5 ml of 100 mM PB, pH 7.0, containing 5 mM $MgCl_2$.

Purification of mutanolysin. The purified fraction of mutanolysin, M1, retained proteolytic activity; therefore, it was necessary to further purify this enzyme. This was accomplished by a two-step procedure (75). The enzyme (15-16 mg) was suspended in H_2O using sonication and then centrifuged at 30,900 x g for 1 h. The supernatant was applied to a carboxymethyl Sephadex C-25 ion exchange column (30 x 0.9 cm; Pharmacia Fine Chemicals, Piscataway, NJ) and eluted with a linear gradient of 0.01 M to 0.15 M phosphate buffer, pH 7.0, (600 ml). The column had been prewashed with 0.01 M PB, 0.15 M PB, and reequilibrated with the starting buffer. Fractions of approximately 5 ml were collected by gravity at a flow rate of 12-15 ml/h. Muralytic activity eluted at 0.045 M to 0.05 M phosphate buffer. Fractions were read at 280 nm in a Gilford 2600 recording spectrophotometer. Those that had an absorbancy

of greater than 0.05 were pooled and concentrated by pressure dialysis. The protein recovery was approximately 50% of starting material. Removal of protease activity was monitored by the Azacoll assay (Calbiochem-Behring, La Jolla CA). The pooled, concentrated material, 125 µg, was incubated with the chromogenic substrate, Azacoll, 10 mg, in 50 mM PB, pH 7.0, to a total volume of 2.5 ml. After 2 h at 37 C, the substrate was centrifuged at 12,000 x g for 10 min and the supernatant was read against a blank tube which had contained Azacoll but no enzyme. That the purification procedure had successfully removed protease activity was determined by an absence of solubilized chromogen.

Mutagenesis and mutant selection. A modification of the methanesulfonic acid ethyl ester (EMS; Sigma Chemical Co.) mutagenesis procedure developed by Shanmugam and Valentine (74) was used to mutagenize a culture of S. mutans. Cells were grown in DM plus 20 mM glucose. At early-log phase (Klett=34), 0.1 ml of EMS was added and the incubation of cells continued for 60 min at 37 C. At the end of this time period, the cells were harvested at ambient temperature and washed once with carbon-free DM and sonicated for 30 sec. The volume was brought up to 10 ml with carbon-free medium and the treated cells were incubated at 48 C for 40 min. The cells were pelleted at ambient temperature and resuspended in a small volume of TYE before sonicating for 30 sec. The volume was brought up to 10 ml with TYE supplemented with 20 mM glucosamine and the culture was incubated until turbidimetric increases, presumed to be growth, resumed. At this time, streptozotocin was added to select for glc PTS^- mutants according to the procedure of Lengeler (33). Streptozotocin was added so that the final concentration of this

glucose analogue was 50 µg/ml and incubation at 37 C proceeded for 5 h. The cells were harvested, washed once with carbon-free medium and then allowed to grow overnight in TYE supplemented with 20 mM lactose. The cells were then transferred to fresh TYE supplemented with glucosamine and at early-log phase (Klett = 35) streptozotocin was added to give a final concentration of 50 µg/ml. After 5 h at 37 C, the cells were harvested, washed once with carbon-free medium, and plated on TYE supplemented with 20 mM lactose plus 20 mM 2-DG. After 48 h, the plates were replicated on TYE containing 20 mM glucosamine or 20 mM lactose. Colonies that grew on lactose but not glucosamine were patched onto lactose/2-DG containing TYE agar. These plates were finally replicated onto TYE plates containing either 20 mM glucosamine or 20 mM lactose. Colonies were chosen which grew only on lactose. These colonies were grown in TYE broth plus 20 mM mannitol and the resultant culture was stored in glycerol at -40 C.

Dry weight determination. Cells were grown in DM plus 50 mM lactose. At various times during their growth cycle, 30 ml portions were removed, harvested, and resuspended in 30 ml, 100 mM PB, pH 7.0, containing 5 mM $MgCl_2$. The absorbancy of this suspension was read at 600 nm in a Gilford 2600 recording spectrophotometer. The cells were collected by centrifugation and resuspended in 3.0 ml of the same buffer. A portion, 0.95 ml, was aliquoted in triplicate to predried (72 h, 60 C) and preweighed aluminum weighing pans. Dry weight (60 C) readings were taken at 24, 48, and 72 h. The readings did not vary and therefore an average value for all three time periods was obtained; and from this average the weight of buffer alone was subtracted to obtain a mean dry weight/absorbancy unit.

Chemical analysis. The origin of the sugars routinely used throughout this study was Sigma Chemical Co. for 2-deoxyglucose (2-DG), mannitol, α-methylglucopyranoside (α-MG), methyl-β-D-thiogalactopyranoside (TMG), galactose, and lactose and Calbiochem-Behring for fructose and mannose. In order to ascertain the degree of purity of these sugars each commercial product was analyzed by gas-liquid chromatography. The sugars were converted to their alditol acetate derivatives by the method of Griggs et al. (15). The derivatives were separated on a glass column (6 ft x 2 mm) packed with 3% SP2330 on Supelcoport 100/120 mesh (Applied Science Lab Inc., State College, PA) on a Tracor model 560 gas chromatograph (Tracor Instruments Inc., Austin, TX). The initial temperature was 180 C and after an initial hold of 5 min, the temperature was raised to 240 C at 2 C/min intervals. The flow rate of the carrier gas, N_2, was 20 ml/min. Detection of the peaks was by a hydrogen flame ionization detector and an Autolab Minigrator electronic digital integrator (Spectra-Physics, Santa Okra, CA) was used to quantitate the peaks.

The amounts of glucose, a common contaminant, were as follows: 2-DG, 0.521%; mannose, 0.0626%; mannitol, 0.184%; galactose, < 0.01%, lactose, 0.0015%. The anomers of fructose and α-MG derivatives have the same retention times as glucose, therefore, quantitation was not possible. TMG was not tested.

The method of Lowry et al. (37) was used for protein determination. To solubilize membranes, 3% sodium dodecyl sulfate (SDS) was added. Proteins added during the purification of membranes (e.g., RNase, DNase, and mutanolysin) were not subtracted from the final data determinations.

Radioisotopes. The following radioisotopes were purchased from New England Nuclear: D-[^{14}C(U)]-glucose (4.0 mCi/mmole); D-[^{14}C(U)]-fructose (359 mCi/mmole); methyl (α-D-[^{14}C(U)]-gluco)pyranoside (275 mCi/mmole); D-[1,2-^{3}H]-2-deoxyglucose (37.3 Ci/mmole); D-[1-^{3}H(N)]-mannitol (17 Ci/mmole); methyl(β-D-[methyl-^{14}C]thiogalacto)pyranoside (54.7 mCi/mmole); D-[1-^{14}C]-mannose (48.6 mCi/mmole); [^{14}C(U)]-lactose (0.97 mCi/mmole); D-[^{14}C(U)]-2-deoxyglucose (282 mCi/mmole). Phosphoenol-[1-^{14}C]-pyruvic acid (10.6 mCi/mmole) was purchased from Amersham (Arlington Hgts., IL).

RESULTS

General conditions for assay of phosphotransferase systems. The initial objectives of this study were concerned with learning about the regulation of lactose uptake in S. mutans GS5. Therefore, many of the basic parameters for the study of the phosphotransferase systems (PT-systems) in this strain were established using the lac-PTS as a model. For these studies, two assays were employed: (1) ONPG plus PEP and ONPG-6-P were used as substrates for the PTS and for phospho-β-galactosidase, respectively, and (2) the generation of pyruvate from the donation of the phosphoryl group of PEP to lactose was measured by the oxidation of NADH in the presence of lactate dehydrogenase (LDH). Since there is a stoichiometric relationship between lactose and PEP and between PEP and NADH, the amount of NADH oxidized is a measure of the amount of lactose phosphorylated.

Fig. 3 demonstrates the linearity of the two PTS assays. Up to 625 μg dry weight of cells may be used to obtain a linear relationship in the utilization of ONPG plus PEP under the conditions outlined in Methods. On the other hand, the oxidation of NADH is linear up to 325 μg of cells, after which a plateau is reached. The measurement of NADH oxidation is approximately 70-fold more sensitive than the phosphorylation and subsequent cleavage of ONPG as determined by comparison of the results obtained by the two different assays using the same dry weight measurement of cells. This may reflect the relative affinities of the lac PTS for ONPG vs. lactose. ONPG, an analogue of lactose, would be expected to have a lesser affinity than the natural substrate.

Fig. 3. Lactose phosphotransferase system activity as a function of cell concentration. Cells were decryptified as described in Methods except the toluene-acetone mixture was 50 µl/ml cell suspension. ONP released by the ONPG + PEP reaction (●) and NAD^+ generated by the LDH/NADH linked-spectrophotometric assay (o) were measured as described in Methods.

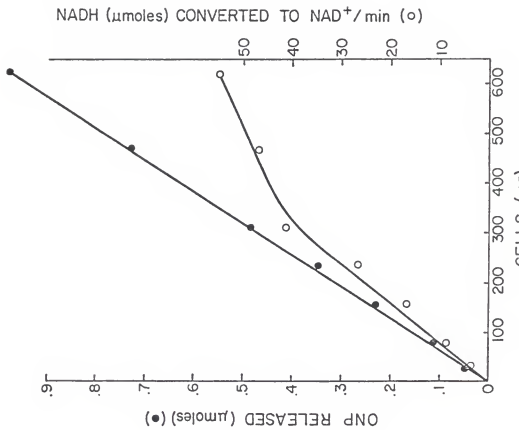

NADH (μmoles) CONVERTED TO NAD+/min (○)
ONP RELEASED (μmoles) (●)
CELLS (μg)
0
100
200
300
400
500
600
.1
.2
.3
.4
.5
.6
.7
.8
.9
10
20
30
40
50

Fig. 4 demonstrates that the pH optimum of the lac PTS is 7.0 using either of the two reaction systems described above. This optimum is dependent on the buffer system used. Table 2 shows the results of assaying for the lac PTS using the two different methods and phospho-β-galactosidase in the presence of various buffers. Phosphate buffer at pH 7.0 is optimal for both PTS and phospho-β-galactosidase activities.

Lac PTS activity appears to be independent of ionic strength between 10 and 500 mM sodium phosphate. This can be seen in Table 3. Also, phospho-β-galactosidase activity is most pronounced at ionic strengths between 50 and 1000 mM. On the other hand, NAD^+ production is independent of all ionic strengths employed.

Assaying whole cells required the permeabilization of membranes to phosphorylated compounds such as PEP using a toluene-acetone mixture. The rationale for using "decryptified" cells was to allow for controlled intracellular concentrations of reactants. Since this type of treatment may cause alterations of the membrane proteins, it was necessary to standardize the conditions of this procedure. Decryptification involves the addition of the toluene-acetone mixture followed by vigorous shaking of the cell suspensions. Table 4 outlines a variety of parameters followed during this step. Two proportions of toluene with acetone were examined: 1:3 and 1:8 (v/v). The amount of this mixture per cell volume was considered as well as the extent of mixing using a Vortex Mixer. All shaking was done at 2-min intervals with approximately 1-min cooling in ice.

A toluene-acetone mixture of 1:3 (v/v) at a final concentration of 100 µl/ml cell culture allowed for the highest lac PTS activity. The

Fig. 4. The pH optimum for the lactose phosphotransferase system. Cells were grown and decryptified as outlined in Table 2. A cell concentration of 120 µg (dry weight) was used to measure ONP released by the ONPG + PEP reaction (●) and NAD^+ generated by the LDH/NADH-linked spectrophotometric assay (○) at indicated pH levels in 60 mM PB (ONP assay) and 70 mM PB (spectrophotometric assay).

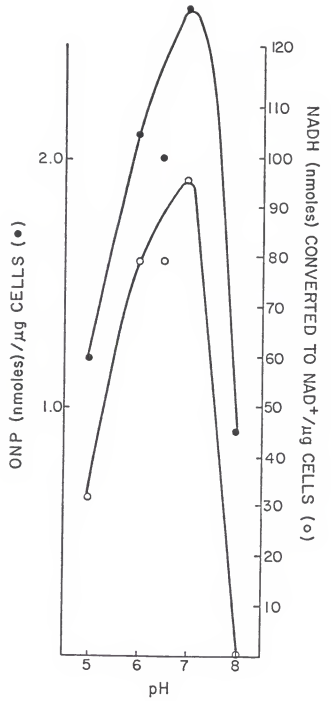

ONP (nmoles)/µg CELLS (●)
NADH (nmoles) CONVERTED TO NAD+/µg CELLS (○)
2.0
1.0
120
110
100
90
80
70
60
50
40
30
20
10
5
6
7
8
pH

Table 2. Lactose phosphotransferase system and phospho-β-galactosidase enzyme activities as a function of buffer composition and pH.

Buffer	pH	nmoles ONP/μg cells[b]		
		ONPG + PEP	ONPG-6-P	μmoles NAD^+/min/μg[b] cells
MES[a]	5.0	.09	1.38	0
	6.0	.43	2.10	27.9
	6.5	.71	4.44	47.9
PB[a]	6.5	.66	4.64	45.0
	7.0	.86	5.25	75.7
	7.5	.81	4.48	67.9
MOPS[a]	7.0	.78	4.23	62.1
TRIS-HCl[a]	7.5	.72	1.80	39.3
	8.0	.59	1.46	45.0
	9.0	.44	1.11	8.5
	10.0	.21	0.18	0

[a]MES: 2-(N-morpholino)ethanesulfonic acid, sodium salt; PB: sodium phosphate; MOPS: 3-(N-morpholino)propanesulfonic acid, sodium salt; Tris-HCl: tris-(hydroxymethyl)-aminomethane hydrochloride.

[b]Cells were grown to late-log/early-stationary phase in defined medium containing 5.0 mM lactose, harvested, washed 1 x with 100 mM PB, pH 7.0, containing 5 mM $MgCl_2$ and then resuspended in 100 mM PB, pH 7.0, containing 5 mM $MgCl_2$ to a final volume $1/20^{th}$ of the original culture. The cells were decryptified with a toluene-acetone mixture at a final ratio of 50 μl/ml of cells for 5 min. All reactants were made up in H_2O. The final concentrations of the above buffers were 70 mM for the LDH/NADH-linked assay, 60 mM for the ONPG + PEP assay, and 80 mM for the ONPG-6-P assay. Since cells were suspended in 100 mM PB, pH 7.0, the contribution of this buffer was minimized by using a 2-fold suspension of cells and one-half the routine volume of cell-suspension. This results in a 2.5 mM concentration of PB in the LDH/NADH assay, 3.8 mM in the ONPG + PEP assay, and 1.8 mM in the ONPG-6-P assay. The concentration of cells was 140 μg, dry weight, for the LDH/NADH-linked and ONPG assays and 56 μg, dry weight, for the ONPG-6-P assay. $MgCl_2$ concentrations were 3.5 mM for the NADH/LDH-linked assay, 3.0 mM for the ONPG + PEP assay, and 4.0 mM for the ONPG-6-P assay. See Methods for details of each enzymatic assay procedure.

Table 3. Lactose phosphotransferase system and phospho-β-galactosidase activities as a function of ionic strength of buffer reagent.

mM[a]	nmoles ONP/μg cells[b]		μmoles NAD^+/min/μg cells[b]
	ONPG + PEP	ONPG-6-P	
1000	.091	4.18	20.9
500	.175	3.82	20.5
100	.138	6.90	22.2
50	.166	5.72	24.0
10	.168	1.70	14.3

[a]PB, pH 7.0.

[b]The procedures used are as outlined in Table 2.

Table 4. Determination of optimal amounts of solvents for decryptification of cells for phosphotransferase enzyme assays.[a]

Toluene-acetone (v/v)	1:3	1:3	1:3	1:3	1:3	1:3	1:8
µl/ml	100	100	100	100	50	25	100
Vortex time (min)	0	2	4	5	5	5	5
ONPG + PEP[b]	.534	.981	.913	.816	.515	.515	.476
ONPG-6-P[c]	21.7	25.7	26.9	26.9	35.0	28.1	36.2

[a]Cells of S. mutans GS5 were cultured overnight in defined medium containing 5.0 mM lactose, harvested, and washed as described in Methods.

[b]Cells, 103 µg (dry weight), were incubated with 100 mM PB, pH 7.0, 5 mM $MgCl_2$, 8 mM PEP, and 8 mM ONPG (total volume: 600 µl) for 30 min at 37 C. The reaction was stopped with cold 5% $NaCO_3$ and the cells were removed by centrifugation. The amount of ONP formed was determined by spectrophotometric measurements of the supernatants at 420 nm. Results are expressed as nmoles ONP/µg cell (dry weight).

[c]Cells, 21 µg (dry weight), were incubated with 100 mM PB, pH 7.0, 5 mM $MgCl_2$, and 100 mM ONPG-6-P (total volume: 500 µl) for 30 min at 37 C. The reaction was determined as above and is expressed as nmoles/µg cells (dry weight).

permeabilization appeared to be complete after 2-min of mixing. If phospho-β-galactosidase activity is measured a somewhat different profile is obtained; optimization occurred at a 50 µl/ml cell culture of a 1:3 (v/v) mixture or a 100 µl/ml cell culture of a 1:8 (v/v) mixture. Since the emphasis of the present study is on the various PT-systems, the optimum conditions were chosen on this basis.

Table 5 compares sugar transport into intact cells vs. phosphorylation of that sugar by decryptified cells. As can be seen with both fructose and glucose, transport results in a 2-fold greater amount of sugar being converted to its phosphorylated derivative compared to phosphorylation. This could be due to one of several factors. First, treatment of the membrane with toluene-acetone may result in an inactivation and/or rearrangement of the transport proteins thereby preventing maximal uptake of sugar for phosphorylation. Second, two glucose transport systems have been shown to be operative in this organism (18). Part or all of this higher transport activity may be due to a contribution of this second system. Since this latter system requires a proton motive force, it can be observed with intact cells only. Also, the presence of an ATP-dependent kinase (see Table 6) indicates an alternate fructose system; therefore, the above discussion concerning glucose transport may pertain to fructose transport. Lastly, this assay was performed with a radiolabelled sugar substrate which is converted to a labelled phosphorylated derivative. The product is separated from the reactant by an anion exchange filter which will trap the negatively-charged sugar phosphate or, alternatively if intact cells are used, the negatively charged cells. These filters may be more efficient for

Table 5. Comparison of sugar (glucose and fructose) transport and phosphorylation by decryptified or untreated cells.[a]

	Substrate	
	Glucose	Fructose
	(nmoles/ml)	
Sugar transported[b]	2.4	1.5
Sugar phosphorylated[c]	1.2	0.7

[a]Cells were grown in fructose according to the procedure outlined in Methods. At early-stationary phase, the cells were harvested and washed 1 x and finally resuspended to 4 x their original concentration. One-half of this suspension was decryptified with a toluene-acetone mixture as described previously and the other half was untreated.

[b]For transport assays, untreated cells (115 μg) were incubated with 40 mM PB, pH 7.0, 2 mM $MgCl_2$, 10 mM NaF, and either 100 μM D-glucose plus 125 μM D-[^{14}C(U)]-glucose (4 μCi/μmole) or 100 μM D-fructose plus 1 μM D-[^{14}C(U)]-fructose (359 μCi/μmole).

[c]Decryptified cells (113 μg) were used to test for sugar phosphorylation. The conditions were altered from the transport assay in the following manner: 10 mM PEP was added and incubation was in 50 mM PB, pH 7.0, plus 2.5 mM $MgCl_2$. The same amounts of radiolabelled substrates were used.

In both types of reactions, incubation was carried out at 37 C for 10 min after which time 0.2 ml was removed and diluted into 2 ml of 1% cold sugar. These were then passed through a DE-81 filter and the filters counted in a liquid scintillation counter. Raw counts were then converted to dpm and nmoles/ml were calculated from known specific activities.

binding the cells than the product, thus, the higher apparent transport activity may be partly due to an inherent characteristic of the assay.

Even though sugar transport appeared to lend itself to a more sensitive assay, I chose to study sugar phosphorylation using decryptified cells. The reasons for this were as follows: (1) the concentrations of the reactants could be controlled more carefully, and (2) the use of decryptified cells eliminated any secondary transport systems (e.g., via the proton motive force) that may be present.

Fig. 5 profiles the lac PTS and phospho-β-galactosidase activities during the growth curve described by strain GS5 when grown on this sugar. It is evident that the lac PTS reaches maximum activity at early-stationary phase. This was confirmed by the LDH/NADH assay (data not shown). The catabolic enzyme, phospho-β-galactosidase, however, reaches maximal levels of activity at early-log phase. This maximization of phospho-β-galactosidase appears to coincide with the early detection of the lac PTS. In all subsequent experiments involving the several PT-systems, late-log or early-stationary cells were used.

Phosphorylation of sugars by the various PT-systems (e.g., glc PTS) is dependent on PEP by definition; ATP will not substitute for PEP. This is clearly seen in Table 6 in the case of glucose. However, ATP was able to donate its phosphoryl group to fructose and this reaction provided 40% the amount of fructose-6-phosphate formed as when PEP was the donor. Mannose was also able to accept a phosphoryl group, but only 8% of product formed as compared to assays using PEP as a phosphoryl source. This organism has been shown to contain a fructokinase which is able to catalyze mannose phosphorylation (5). Such an enzyme

Fig. 5. Lactose phosphotransferase system enzyme activities and growth of S. mutans GS5 as a function of time. Cells were grown in defined medium plus 5.0 mM lactose over a period of 7 h and turbidities measured using the Klett (●). At defined intervals, aliquots were removed and cells decryptified for the ONPG + PEP assay releasing ONP (○) and the ONPG-6-P assay releasing ONP (Δ). The latter assay measures phospho-β-galactosidase activity.

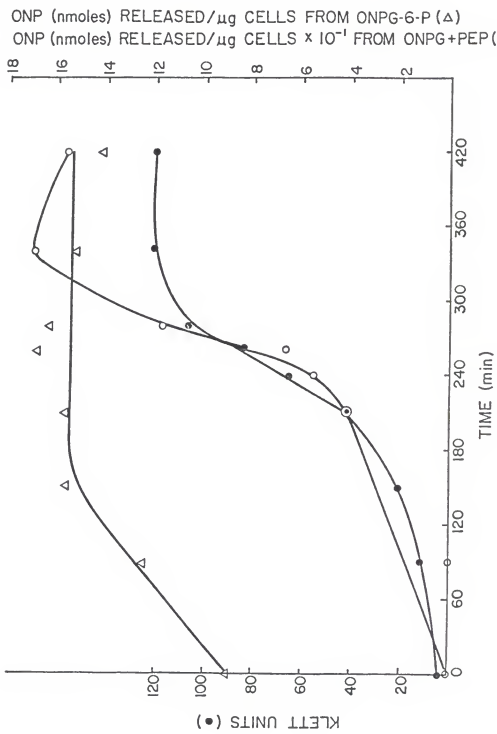

ONP (nmoles) RELEASED/µg CELLS FROM ONPG-6-P (△)
ONP (nmoles) RELEASED/µg CELLS x 10⁻¹ FROM ONPG+PEP (○)
KLETT UNITS (●)
TIME (min)
0
60
120
180
240
300
360
420
20
40
60
80
100
120
2
4
6
8
10
12
14
16
18

Table 6. Determination of phosphoenolpyruvate dependency for phosphotransferase system-mediated phosphorylation.

	Substrates for phosphorylation		
	Glucose	Fructose	Mannose
ATP	0[a]	0.5[a]	0.06[b]
PEP	2.9[a]	1.2[a]	0.77[b]

[a]Cells grown in defined medium plus 5 mM glucose were harvested, washed, and decryptified. The permeabilized cells, 153 µg (dry weight) were incubated with 70 mM PB, pH 7.0, 3.5 mM $MgCl_2$, 10 mM ATP or PEP, 10 mM NaF and 100 µM D-glucose plus 125 µM D-[^{14}C(U)]-glucose (4 µCi/µmole) or 100 µM D-fructose plus 1 µM D-[^{14}C(U)]-fructose (359 µCi/µmole). The final reaction volume was 1.0 ml.

[b]Cells grown in defined medium plus 5 mM glucose were harvested, washed, and decryptified. The permeabilized cells, 109 µg (dry weight) were incubated with 70 mM PB, pH 7.0, 3.5 mM $MgCl_2$, 10 mM ATP or PEP, 10 mM NaF, and 100 µM D-mannose plus 10 µM D-[1-^{14}C]-mannose (48.6 µCi/µmole). The final reaction volume was 1.0 ml.

In both a and b, the reaction was allowed to proceed for 10 min at 25 C. Reactions were halted when 0.1 ml was removed and diluted in 1% homologous sugar, filtered through a DE-81 filter and counted in a liquid scintillation counter. Results are expressed as nmoles sugar phosphorylated/ml.

is likely responsible for the small amount of phosphorylation seen here. The fructokinase is present in glucose-, mannose- and fructose-grown cells (data not shown). In all experiments performed in this study PEP was used, however, the possibility exists that some PEP was converted to ATP via pyruvate kinase. All results using fructose as a substrate are interpreted as being at least 60% dependent on the PTS. Fig. 6 demonstrates that at the concentration used in this study, 10 mM, PEP is well beyond the limiting range. Resting cells of S. lactis have been shown to contain an energy reserve in the form of 3-phosphoglycerate (86). Presumably, S. mutans cells in early-stationary phase contain a similar reserve since transport in intact cells is observed (Table 5). If NaF is excluded, glucose phosphorylation occurs in decryptified cells without the addition of PEP (data not shown). Reproducibility of this result was not always obtained, an observation confirmed by others (Dr. G. Jacobson, personal communication). Therefore, for purposes of standardization, NaF was always included and the concentration used, 10 mM, gave maximum inhibition of glucose phosphorylation in the absence of PEP (data not shown).

Comparative study of phosphotransferase-mediated sugar phosphorylation. In order to obtain a profile of the various PT-systems in this organism, a survey was set up in which the cells were grown in various growth substrates and assayed for the presence of specific PT-systems. The results are outlined in Table 7. The glc PTS and man PTS can be detected under all the growth conditions tested leading to the conclusion that these two sugars are phosphorylated via a constitutive system. Phosphorylation of 2-deoxyglucose (2-DG) was detected in all cells

Fig. 6. Glucose phosphotransferase system activity as a function of phosphoenolpyruvate (PEP) concentration. Cells were grown in defined medium plus 5 mM glucose and decryptified as in Methods. Cells(63 µg dry weight), were incubated with varying concentrations of PEP plus 25 µM D-[^{14}C(U)]-glucose (4 µCi/µmole) and 100 µM D-glucose in 25 mM PB, pH 7.0, containing 1.3 mM $MgCl_2$. Product (glucose-6-phosphate) was measured by filtering cells on DE-81 filters and counts retained on filters were measured and converted to dpm and subsequently to nmoles/ml reaction mixture.

GLUCOSE-6-PHOSPHATE (nmoles/ml)
2.4
2.0
1.6
1.2
.8
.4
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
PEP (mM)

Table 7. Induction of phosphoenolpyruvate-dependent phosphotransferase systems as a function of carbon source in growth media (LDH/NADH-linked assay).

Substrate tested[b]	Growth carbon source[a]				
	Glucose	Mannose	Mannitol	Lactose	Galactose
Glucose	6.3	8.1	12.7	1.5	18.3
Mannose	4.5	4.9	4.8	1.9	15.7
Mannitol	0	0	3.0	0	0
2-Deoxyglucose	0.9	0.9	2.0	0	9.4
α-Methylglucoside	0	0	0	0	0
Galactose	0	0	0	0	0
Isopropyl-β-D-thiogalactopyranoside	0	0	0	1.4	3.9
Lactose	0	0	0	7.5	20.7
Glucosamine	5.5	N.T.[c]	N.T.	N.T.	N.T.

[a]Cells were grown in the several sugars as described under Methods. After harvesting and washing, they were resuspended in buffer to $1/20^{th}$ of their original volumes. The cell concentration varied between 60-325 μg/ml. To insure linearity, at least two cell concentrations were used to test a given sugar for sugar phosphorylation. Where activity could not be detected the upper limits of this range (240-325 μg) were reported.

[b]The LDH/NADH-linked spectrophotometric assay was used to assay for sugar phosphorylation as described in Methods. All data are expressed as μmoles NADH converted to NAD^+/μg cells/min x 10^{-2}.

[c]Not tested.

except those grown in lactose. This compound is a glucose analogue and thus would be expected to be transported via the glc PTS as is the case in E. coli (21). Schachtéle and Mayo (71) showed that the uptake and phosphorylation of this analogue in S. mutans is through a PTS, most likely the glc PTS. The detection of PEP-dependent phosphorylation of this sugar analogue in all but lactose-grown cells argues for such a PTS being constitutive. The most likely reason for its absence in lactose-grown cells is that low levels of the glc PTS preclude the detection of 2-DG phosphorylating activity given the sensitivity of the assay used. In agreement with Schachtele and Mayo (71), mannitol-grown cells show a high level of 2-DG activity; again, most likely because glc PTS activity is high in these cells.

One means of testing for the presence of two glc PT-systems is to compare the phosphorylation of the two glucose analogues, 2-DG and α-MG (21). It can be seen (Table 7) that α-MG is not phosphorylated, thereby leading to the tentative conclusion that there is only one glucose system operating in this organism and it would be analogous to the low affinity system in E. coli. Mannitol, as has been shown by others (40), is phosphorylated by an inducible system. Interestingly, galactose is not an apparent PTS substrate even though growth in this sugar leads to a high level of other PT-systems. This may be related to the poor growth of the cells in this carbon source. Lactose and its analogue, isopropyl-ß-D-thiogalactopyranoside (IPTG) appeared to be phosphorylated by an inducible system. This is in agreement with a report published by Hamilton and Lo(17). Galactose as well as lactose appear to be inducers; in S. aureus, galactose-6-phosphate has been shown to be the inducer for the lac PTS (43).

The results obtained in Table 8 reaffirm the general pattern discussed above. Here the cells were grown as in Table 7 but the PTS assay was conducted using radiolabelled substrate at a saturating level. Glucose, again, appears to be constitutive. The relative levels are similar to those found using the LDH/NADH assay. Glc PTS is low in lactose-grown cells and high in mannitol-grown cells. However, there appears to be a contradiction with mannose-grown cells in that PEP-dependent glucose phosphorylating activity is lower than anticipated. In agreement with the previous results, 2-DG is phosphorylated while α-MG is not. Mannose phosphorylation does not vary greatly between mannose- and glucose-grown cells but shows a significant decrease in lactose-grown cells. Fructose, like glucose and mannose, appears to be phosphorylated by a constitutive system.

The constitutive nature of PEP-dependent phosphorylation of glucose, mannose, and fructose suggests a possible physiological relationship amongst the PT-systems for these three sugars. As discussed in the Introduction, the low affinity glc PTS in E. coli phosphorylates glucose, mannose, fructose, and glucosamine (31). In addition, this system phosphorylates 2-DG but not α-MG. Since it appeared that an analogous system existed in this strain of S. mutans, a series of competition experiments was done to test for this possibility. In these experiments, a ^{14}C-substrate competed for phosphorylation against an excess of unlabelled sugar. The results are presented in Table 9. Glucose and mannose were mutually competitive. Mannose was a less efficient competitor for glucose phosphorylation than was glucose. Glucosamine was also a competitor for both mannose and glucose but at a lower degree of

Table 8. Induction of phosphoenolpyruvate-dependent phosphotransferase systems as a function of carbon source in growth media (radioactive assay).

Substrate tested[b]	Growth carbon source[a]			
	Glucose	Mannose	Mannitol	Lactose
	(pmoles sugar-phosphate/μg cell dry weight)			
Glucose	28.7[c]	18.5	95.8	38.6
Mannose	44.0	30.5	N.T.	6.4
2-Deoxyglucose	0.9	N.T.	N.T.	N.T.
α-Methylglucoside	0	N.T.	N.T.	N.T.
Methyl-β-D-thiogalactopyranoside	N.T.[d]	N.T.	N.T.	3.5
Mannitol	N.T.	0	12.3	N.T.
Fructose	56.1	20.0	49.3	4.8

[a]Cells were grown as described previously (Table 5, Methods).

[b]Cells were decryptified and assayed with various sugars. The final concentrations of reactants were: 30 mM PB, pH 7.0, 1.5 mM $MgCl_2$, 10 mM PEP, and 10 mM NaF. The substrate concentrations included 100 μM of unlabelled sugar containing the following concentrations of the homologous radioactive substrates: 50 μM D-[^{14}C(U)]-glucose (4 μCi/μmole), 3 μM D-[1-^{14}C]-mannose (48.6 μCi/μmole), 0.03 μM D-[1,2-^{3}H]-2-deoxyglucose (37.3 mCi/μmole), 0.08 μM methyl-α-D-[^{14}C(U)]-glucoside (275 μCi/μmole), 19 μM [methyl-^{14}C]-β-D-thiogalactopyranoside (54.7 μCi/μmole), 0.006 μM D-[1-^{3}H(N)]-mannitol (17 mCi/μmole) or 2 μM D-[^{14}C(U)]-fructose (359 μCi/μmole) Reaction mixtures included a range of 12-350 μg cells and specific activities were calculated from an average of those values falling within the linear portion of the curves generated (not shown).

[c]The standard deviation for 5 identical samples was ± 3.8.

[d]Not tested.

Table 9. Competitive inhibition by unlabelled sugars of uptake of radiolabelled sugars by S. mutans GS5.[a]

Radiolabelled substrates[b]	Glucose	Mannose	Glucosamine	α-Methylglucoside	Fructose	Mannitol	Methyl-β-D-galactopyranoside
				(% Inhibition)			
D-[1-^{14}C]-Mannose	96	93	67	N.T.[c]	39	N.T.	N.T.
D-[^{14}C(U)]-Glucose	99	74	54	22	1	0	8
	*89	20	0	0	0	0	0
D-[^{14}C(U)]-2-Deoxyglucose	94	95	86	N.T.	28	N.T.	17
D-[^{14}C(U)]-Fructose	0	25	0	0	99	0	0

[a]Cells were grown in defined medium plus 5.0 mM glucose and prepared as described in Methods.

[b]The assay contained 30 mM PB, pH 7.0; 1.5 mM $MgCl_2$; 10 mM PEP; 10 mM NaF; 0, 2 (shown by asterisk), or 10 mM competing unlabelled sugars; and cells, 85-100 ug (dry weight). The ^{14}C-sugar concentrations were: glucose, 250 μM; fructose, 1.5 μM; mannose, 10.5 μM; and 2-deoxyglucose, 3.5 μM. The incubation mixture was held at 25 C for 9 min and then diluted with a cold 1% solution of the homologous sugar. This was held at 4 C until filtered through a DE-81 filter. Results are expressed as percent inhibition by 2 or 10 mM unlabelled sugar of uptake of labelled sugar.

[c]Not tested.

efficiency. Glucose phosphorylation appeared to be inhibited by α-MG but since direct phosphorylation of this analogue could not be demonstrated, it was concluded that this observed inhibition was the result of a non-specific mechanism. As expected, fructose competed with its ^{14}C-isotope; however, it did not inhibit glucose phosphorylation. There was a mutual competition between mannose and fructose. This may be due to some recognition of mannose by a frc-PTS or the sharing of a distinct "mannose site" on the glc PTS with fructose. If the latter explanation is correct, affinity for fructose would be extremely low since this sugar does not interfere with glucose activity. Alternatively, this mutual reaction may reflect the action of a manno-fructokinase. It has been shown that S. mutans possesses an ATP-dependent kinase capable of recognizing both fructose and mannose (5). ATP may be generated by pyruvate kinase and this may be available in PEP-supplied cells. Table 6 indicates a role for ATP in sugar phosphorylation. However, this mechanism would not account for all the inhibition observed. As would be predicted, mannitol and methyl-β-D-thiogalactopyranoside (TMG) do not inhibit the reactions seen with glucose or fructose.

In addition, both glucose and mannose, as well as glucosamine, inhibit 2-DG phosphorylation. Fructose is slightly inhibitory, however TMG also shows some competitive inhibition which indicates a degree of non-specificity to the reaction. If a "mannose site" exists on the glc PTS and 2-DG shows some affinity for this site, then one may postulate that this is the site where fructose is inhibiting. Since 2-DG is contaminated with glucose (see Methods), it is difficult to interpret the data shown in Table 9 using this glucose analogue as a competitive

inhibitor. Calculations showed that the amount of inhibition of glucose phosphorylation could be attributable to the contaminating glucose. However, PEP phosphorylation of mannose and fructose in the presence of 2-DG showed 15% and 100%, respectively, of control levels, clearly indicating competition in the case of mannose. Also, the homologous system showed 94% inhibition.

Kinetics of phosphotransferase activities and relative growth rates in various sugars. It was of interest to deduce the relative affinities of the glc/man PTS for substrates. Kinetic analyses were performed and are illustrated in Figs. 7-9. The linear transformation by a double reciprocal plot was calculated by a linear regression analysis. The linear coefficient in the case of glucose was calculated to be .999. The K_m for glucose phosphorylation was calculated from the x-intercept and was found to be 64 μm and the V_{max} which was calculated from the y-intercept was found to be .366 nmoles glucose-6-phosphate formed/min/ml (Fig. 7). For mannose, the linear coefficient was calculated to be .987. The K_m for mannose phosphorylation was calculated to be 90 μm and the V_{max} was .300 nmoles mannose-6-phosphate formed/min/ml (Fig. 8). Not shown is the kinetics of 2-DG phosphorylation, the K_m of this reaction was calculated to be 154 μm.

Fig. 9 shows the results of a kinetic analysis using fructose as a substrate. The K_m of this reaction is 42 μm and the V_{max} is .176 nmoles fructose-6-phosphate formed/min/ml. The linear coefficient in this case is .998.

These relative affinities are reflected in the growth rates in these different carbon sources (Fig. 10). A transformation of these

Fig. 7. Kinetics of glucose-6-phosphate formation by the PEP-dependent phosphotransferase system of S. mutans GS5. Decryptified cells (104 µg, dry weight) obtained from cultures of strain GS5 grown in defined medium plus 5 mM glucose were suspended in a reaction mixture (1.0 ml total volume) of 30 mM PB, pH 7.0, containing 1.5 mM $MgCl_2$, 10 mM PEP, 10 mM NaF, and various concentrations of D-glucose each containing D-[^{14}C(U)]-glucose (0.8 µCi/µmole). After 10 min at 25 C, reactions were stopped by dilution of 0.1 ml of the mixture into 1.0 ml 1% glucose. Reactants were separated from products by a DE-81 filter. Glucose-6-phosphate was measured (●) from counts converted to dpm and then expressed as nmoles/min/ml. A double reciprocal plot (inset) was determined by linear regression analysis. Kinetic data are included in the text.

GLUCOSE-6-PHOSPHATE (nmoles/min/ml)
.30
.25
.20
.15
.10
.05
0
100
200
300
400
500
600
700
800
900
1000
GLUCOSE (μM)
V/S
120
100
80
60
40
20
-10
0
.10
.20
.30
.40
.50
.60
.70
V/S

Fig. 8. Kinetics of mannose-6-phosphate formation by the phosphoenolpyruvate-dependent phosphotransferase system of S. mutans GS5. Decryptified cells (103 µg, dry weight), obtained from cultures grown in defined medium plus 5 mM glucose, were suspended in a reaction mixture (1.0 ml total volume) of identical composition to that employed in Fig. 5 except for the substitution of various concentrations of D-mannose each containing D-[1-^{14}C]-mannose (2.3 µCi/µmole). Reactions were carried out as in the previous experiment (Fig. 5) and stopped by dilution into 1% mannose. Mannose-6-phosphate was measured (●) from counts converted to dpm and then expressed as nmoles/min/ml. A double reciprocal plot (inset) was determined by linear regression analysis. Kinetic data are discussed in the text.

MANNOSE-6-PHOSPHATE (nmoles/min/ml)
MANNOSE (μM)
0
100
200
300
400
500
.1
.2
.3
.4
v/s
-.10
0
.10
.20
.30
.40
v
20
40
60
80
100
120
140

Fig. 9. Kinetics of fructose-6-phosphate formation by the phosphoenol-pyruvate-dependent phosphotransferase system of S. mutans GS5. Decryptified cells (100 µg, dry weight) obtained from cultures grown in defined medium plus 5 mM glucose, were suspended in the reaction mixture employed in Figs. 5 and 6, except for the use of various concentrations of D-fructose, each containing D-[^{14}C(U)]-fructose (5.4 µCi/µmole). Cold 1% fructose was employed to stop reactions. Fructose-6-phosphate was measured (●) from counts converted to dpm and then expressed as nmoles/min/ml. A double reciprocal plot (inset) was done as before and kinetic data are presented in the text.

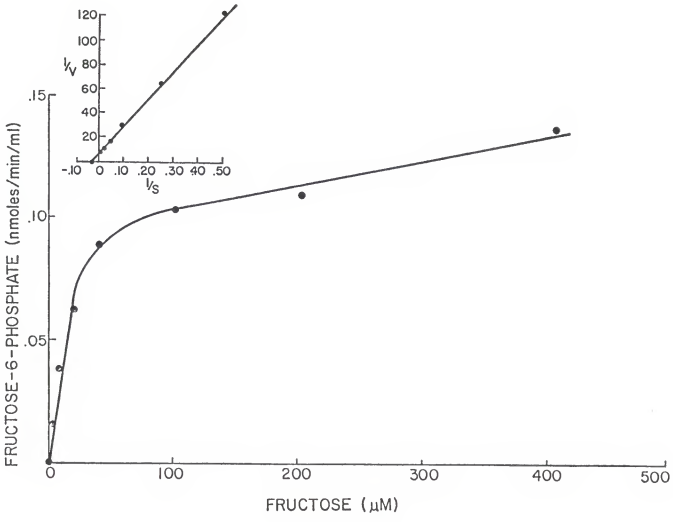
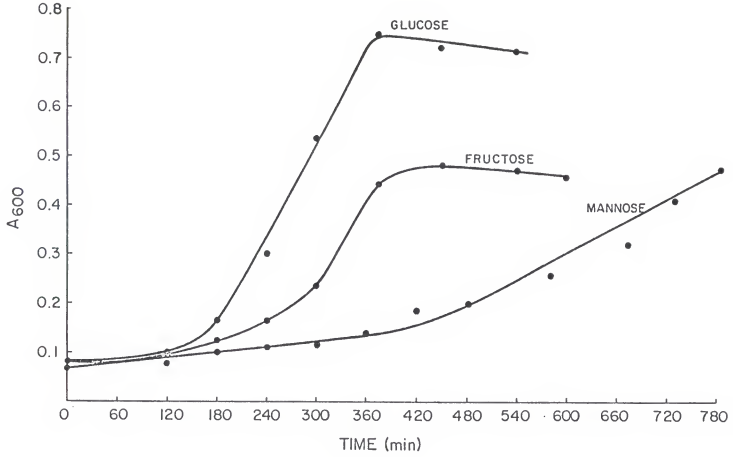

FRUCTOSE-6-PHOSPHATE (nmoles/min/ml)
.15
.10
.05
0
100
200
300
400
500
FRUCTOSE (μM)
1/V
120
100
80
60
40
20
-.10
0
.10
.20
.30
.40
.50
1/S

Fig. 10. Growth of S. mutans GS5 in glucose, fructose, and mannose. Cells were grown for 12 h in TYE plus 20 mM concentrations of either glucose, fructose, or mannose. Cultures (5% inocula) were then transferred to 50 ml defined medium containing 5 mM concentrations of the homologous sugars. At specified times, 10 ml aliquots were removed for absorbancy measurements at 600 nm.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A600
GLUCOSE
FRUCTOSE
MANNOSE
0
60
120
180
240
300
360
420
480
540
600
660
720
780
TIME (min)

data into a semi-log plot allowed the calculation of the mean generation times. The mean generation time in defined medium containing glucose is 75 min; whereas in mannose it is 290 min. With mannose as the growth substrate, a maximum culture density was not achieved after 12 h of growth. However, cultures grown in glucose reach their maximum levels between 5.5 h and 6.0 h under the conditions employed.

The mean generation time using fructose as a carbon source was calculated to be 121 min and maximum growth was achieved in approximately 6.5 h.

Selection of glucose phosphotransferase negative mutants. Data presented thus far indicate that S. mutans GS5 appears to possess a relatively non-specific PTS for glucose uptake. In order to further study the former system, glc PTS negative mutants were selected on the basis of their inability to grow on glucosamine. Wild-type cells non-induced for the lac PTS are unable to grow in the presence of 2-DG since this non-metabolizable glucose analogue is transported by a constitutive system, thereby exhausting the PEP reserves. In addition, evidence will be presented below that suggests glucose represses the induction of the lac PTS. By selecting for a 2-DG resistant mutant, one may be selecting for a glc PTS-negative mutant, since 2-DG is transported by the glc PTS. However, the majority of the 2-DG resistant clones picked appeared to possess an altered glc PTS allowing them to transport and therefore grow on glucose (data not shown). In order to select true glc PTS-negative mutants, a second criterion, the inability to grow on glucosamine was used. However, glucosamine negative cells are not necessarily transport mutants. Clones which

grew on lactose plus 2-DG but not on glucosamine were isolated. A priori, one would expect a mutant selection based on these two criteria to provide glc PTS-negative mutant clones. In addition, glc PTS-negative cells were enriched by incubation in streptozotocin in the step prior to plating (see Methods). After mutagenesis, the cells were allowed to recover in glucosamine. As shown in Table 7, this is a PTS substrate which is carried by the glc PTS (Table 9). Streptozotocin then was added to these cells. Since it is a glucose analogue containing a nitroso group and is carried by the glc PTS (33), those cells still containing an active glc PTS will transport this toxic analogue and should be killed.

Streptozotocin-resistant clones were chosen and allowed to grow in mannitol overnight. A survey was done to determine if the lesion indeed was in the glc PTS and the extent of loss of phosphorylating functions. In order to perform a general survey, the LDH/NADH-linked assay was used. The PTS substrates studied were glucose, mannose, fructose, and mannitol. Glucose, mannose, and fructose were used to determine what, if any, linkage exists between the PTS-mediated phosphorylations of these three sugars and mannitol phosphorylation was assayed as a positive control, since all cells were grown on this substrate. The results which are expressed as percent wild-type activity can be seen in Tables 10-12. Table 10 shows a representative listing of clones which grouped into what was labelled as Group I. These cells could not phosphorylate glucose or mannose; whereas fructose and mannitol phosphorylations were variable but did not correlate with the absence of glucose/mannose phosphorylation. Table 11 shows representatives of Group II. This group had much reduced glucose activity but with one exception mannose

Table 10. Glucose phosphotransferase system negative mutants: Group I.[a]

Clone	Sugar substrate for transport assay[b]			
	Glucose	Mannose	Fructose	Mannitol
3A	0[c]	0	175	94
2A	0	0	125	35
4B	0	0	95	20
12B	0	0	54	24
18B	0	0	42	59
16B	0	NT[d]	50	0
23A	0	0	26	0

[a]Mutants were selected as outlined in Methods.

[b]Cells were grown overnight in TYE broth plus 20 mM mannitol, harvested, washed, and decryptified. The LDH/NADH-linked spectrophotometric assay for sugar phosphorylation was run on each clone using the indicated sugars as described in Methods. In each case a 1 mM sugar concentration was employed.

[c]Results are expressed as percent wild-type.

[d]Not tested.

Table 11. Glucose phosphotransferase system negative mutants: Group II.[a]

Clone	Sugar substrate for transport assay[b]			
	Glucose	Mannose	Fructose	Mannitol
34B	8[c]	0	83	45
8B	13	0	46	48
25B	16	0	104	42
26B	18	0	86	18
4A	20	0	NT[d]	105
10A	21	0	71	100
26B	21	7	129	59
28B	29	0	88	31

[a]Mutants were selected as outlined in Methods.

[b]See Table 9 for assay conditions.

[c]Results are expressed as percent wild-type.

[d]Not tested.

Table 12. Glucose phosphotransferase system negative mutants: Group III.[a]

Clone	Sugar substrate for transport assay[b]			
	Glucose	Mannose	Fructose	Mannitol
49B	30[c]	21	155	97
23B	33	15	117	63
11B	34	15	75	164
13A	49	17	NT[d]	176
46B	38	0	158	33

[a]Mutants were selected as outlined in Methods.

[b]See Table 9 for assay conditions.

[c]Results are expressed as percent wild-type.

[d]Not tested.

activity is totally absent. Fructose phosphorylation was variable but always detectable. Group III clones are listed in Table 12. These cells were characterized by having up to 50% wild-type activity for glucose phosphorylation and, with one exception, residual mannose activity. Fructose activity was near to or above wild-type activity in all mutants which grouped in this category. These data suggest a glc/man PTS and a distinct frc PTS.

Studies of cell-free membranes for phosphotransferase related activities. The study of the glc PTS using isolated membrane fractions was undertaken for a number of reasons. The first reason was to obtain a more detailed picture of this transport system at the molecular and cellular levels. Second was to compare membranes with whole cells in order to evaluate the ability to maintain the native protein structure during the fractionation procedure; specifically when using the muralytic enzyme, mutanolysin (M1), to remove the cell wall. Lastly, and most significantly, was to determine the site(s) of lesion(s) of the mutants described in the previous section. The latter studies are described in the following section. Since the primary focus of this study is the glc PTS, all membranes were prepared from glucose-grown cells.

Mutanolysin (M1) treatment yielded a more active membrane preparation than when mechanical means were used to break cells. The specific activity of glucose phosphorylation using 50 μM D-[^{14}C(U)]-glucose by M1-prepared cell membranes was 14.7 pmoles/μg protein whereas "membranes" obtained from cells broken by glass beads was 4.6 pmoles/μg protein. The M1-derived membranes were 3.2-fold more active than the wall-membrane complexes obtained from cells broken by glass beads in a Bead Beater.

Since a different glucose concentration (25 μM) was used to assay wall-membrane complexes obtained from cells broken in the Braun Tissue Homogenizer, it is more difficult to obtain an exact comparison. However, the results showed a specific activity of 2.3 pmoles/μg protein which again is clearly less than that obtained with M1-prepared membranes. For this reason, all studies involved M1-prepared membranes.

Since PT-systems have two sets of reactions, one that involves soluble proteins and a second that involves the membrane proteins, it was of interest to determine if membranes prepared with M1 would show a requirement for cytoplasmic constituents to carry out the phosphorylation of glucose. The results are outlined in Table 13. It is apparent that the membranes alone are sufficient to carry out a PEP-dependent phosphorylation of glucose. The cytoplasmic material appears to be slightly inhibitory perhaps due to phosphatases present in the soluble fraction. These data should not be interpreted to mean that unlike the majority of PT-systems studied to date only membrane proteins are involved in glucose phosphorylation. As will be discussed below, cytoplasmic constitutients are quite likely trapped within membrane vesicles or perhaps these "cytoplasmic" constituents interact with the membrane as transitory peripheral proteins.

Fig. 11 demonstrates the proportionality of the PEP-dependent phosphorylation of glucose with added membranes. This proportionality exists up to 160 μg of membrane protein. There appears to be some saturability indicating that at least one of the PTS proteins is present in a rate-limiting amount.

Table 13. Glucose phosphorylation by cell fractions of S. mutans GS5.[a]

Fraction[b]	CPM
Cytoplasm	0
Membrane	2025
Cytoplasm + Membrane	1160

[a]Cells, previously grown in defined medium plus 5.0 mM glucose, were fractionated to yield membranes and cytoplasm as described in Methods. The mutanolysin (M1) procedure was employed for this purpose.

[b]Reaction mixtures (final volume: 200 µl) consisted of 80 mM PB, pH 7.0, 4 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 10 mM PEP, 10 mM NaF, 25 µg membrane protein, 114 µg cytoplasmic protein, and 12.5 µM D-[^{14}C(U)]-glucose. The reactants were incubated for 30 min at 37 C after which time they were diluted with excess sugars and the count of radioactivity incorporated into phosphorylated products was determined as described in Methods.

Fig. 11. Phosphorylation of glucose by cell-free membranes. Cell-free membranes, obtained by the mutanolysin procedure (see Methods), were assayed to determine the optimal protein concentration for maximal glucose phosphorylation. Reaction mixtures containing 85 mM PB, pH 7.0, 4 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 10 mM PEP, 175 μM D-[^{14}C(U)]-glucose (4.0 μCi/μmole) and the several membrane concentrations (total volume, 200 μl) were incubated at 37 C for 30 min. Labelled glucose-6-phosphate was separated from labelled glucose by filtration and the filters were measured for radioactivity. Data are expressed as nmoles product/ml.

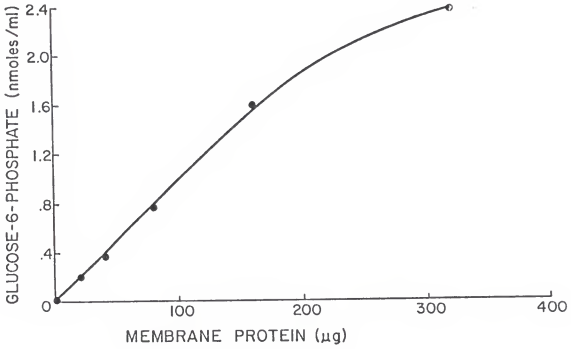

GLUCOSE-6-PHOSPHATE (nmoles/ml)
2.4
2.0
1.6
1.2
.8
.4
0
100
200
300
400
MEMBRANE PROTEIN (µg)

Table 14 demonstrates the PEP requirement to catalyze glucose phosphorylation by cell-free membranes and also, the inability of ATP to substitute for this compound in the PTS reaction. The omission of NaF from the reaction yields a slightly higher concentration of product than when it is included. The difference between these two results yields a result close to that obtained when both PEP and NaF are excluded. However since PEP is in saturating amounts this difference may not be significant. The detection of activity in the absence of both PEP and NaF indicates a residual energy reserve. The inclusion of NaF in the absence of PEP (last line, Table 14) supports this conclusion. These data suggest cytoplasmic contamination of the M1-prepared membranes.

As with whole decryptified cells grown in glucose, membranes prepared from glucose-grown cells are able to carry out the PEP-dependent phosphorylation of glucose, mannose, and fructose (Table 15). These specific activities simply illustrate that these membranes reflect the constitutivity of certain phosphotransferase systems found in the whole decryptified cells. No attempt was made to quantify the loss of activity during membrane preparation due to the complexity of the systems under investigation.

The results of competition experiments using an excess of unlabelled sugar vs. D-[^{14}C(U)]-glucose are described in Table 16. The pattern is similar to that obtained with decryptified cells. Glucose competes with itself. Mannose and 2-DG appear to compete with glucose phosphorylation by membranes more efficiently than in whole cells; however, this may be a reflection of the lower concentration of labelled glucose used in studies with membranes (250 μM vs. 125 μM). In both cells and membranes,

Table 14. Phosphoenolpyruvate-dependent phosphorylation of glucose by mutanolysin-prepared membranes of S. mutans GS5.[a]

Conditions	pmoles glucose-6-phosphate formed/μg protein[b]
+PEP, +NaF	7.3
+PEP, -NaF	8.6
-PEP, -NaF	1.1
+ATP, +NaF	0

[a]Cells were grown in defined medium plus 5.0 mM glucose and membranes were prepared using mutanolysin as described in Methods.

[b]Membranes, 40 μg, were assayed for phosphorylating activity in the presence of 80 mM PB, pH 7.0, 4.0 mM $MgCl_2$, 4.0 mM 2-mercaptoethanol, 10 mM NaF, 10 mM PEP or 10 mM ATP, and 175 μM D-[^{14}C(U)]-glucose (4.0 μCi/μmole) in a total volume of 200 μl. After 30 min at 37 C, the mixture was diluted in 1% cold glucose. Labelled glucose-6-phosphate was measured after filtration of products through a DE-81 filter and expressed as pmoles/μg membrane protein.

Table 15. Phosphotransferase activities of mutanolysin-prepared membranes.[a]

Sugar substrate	Sugar-phosphate produced[b]
	pmoles/μg cell dry weight
Glucose	12.2
Mannose	12.2
Fructose	12.5

[a]Membranes were prepared from glucose-grown cells by the mutanolysin procedure (see Methods).

[b]Membranes were incubated in 85 mM PB, PH 7.0, 4 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 10 mM PEP, and sugar. The sugar concentrations were as follows: 175 μM D-[^{14}C(U)]-glucose (4 μCi/μmole), 100 μM D-mannose plus 10 μM D-[^{14}C]-mannose (48.6 μCi/μmole), or 100 μM D-fructose plus 1 μM D-[^{14}C(U)]-fructose (359 μCi/μmole). In order to obtain a measurement within the linear range two membrane concentrations were used (24 and 48 μg). The reaction mixture (total volume: 200 μl) was incubated at 37 C for 30 min after which time excess cold sugar was added to stop the reaction. Product was collected onto a DE-81 filter for counting in a liquid scintillation counter. Data are expressed as pmoles sugar-phosphate/μg protein.

Table 16. Inhibitory effect of competing sugars on the phosphorylation of D-[^{14}C(U)]-glucose by the phosphoenolpyruvate-dependent phosphotransferase system of decryptified cells and mutanolysin-membranes[a] derived from S. mutans GS5.

Competing sugar	% Inhibition	
	Membranes[b]	Cells[c]
None	0	0
Glucose	100	99
Mannose	93	74
2-Deoxyglucose	86	54
Fructose	7	1
Mannitol	0	0

[a]Membranes were prepared by mutanolysin-induced lysis of glucose-grown cells (see Methods).

[b]To assay membranes, the reaction mixture (final volume: 200 µl) contained 50 mM PB, pH 7.0, 2.5 mM $MgCl_2$, 2.5 mM 2-mercaptoethanol, 10 mM PEP, 10 mM NaF, 0 or 10 mM competing sugar, 125 µM D-[^{14}C(U)]-glucose, and 50 µg membranes. Incubation was carried out at 37 C for 30 min. The amount of radioactivity adhering to DE-81 filters was determined as described in Methods. Results were calculated from the percent decrease in cpm adhering in samples with unlabelled sugar as compared to cpm adhering in samples without unlabelled sugar (percent inhibition).

[c]Data presented in Table 9.

fructose does not compete with glucose phosphorylation. It is evident, therefore, that membranes prepared by mutanolysin treatment mimic the cells from which they are derived; that is, it does not appear that the binding site(s) of the glc PTS has been altered.

The transphosphorylation reaction was first described by Saier et al. (59) as a means of assaying for EII. As described, the EII^{glc} catalyzes the phosphoryl exchange between glucose-6-phosphate and glucose. The ability of membranes to carry out this reaction was studied in order to determine the integrity of the EII^{glc} in cell-free membranes. The reaction is known to be very sensitive to substrate inhibition and requires a ratio of phosphoryl donor to acceptor of 1000:1 to provide an optimum reaction. Table 17 outlines the optimization of the reaction with respect to substrate concentrations. The ratio of donor to acceptor was always 1000:1. The maximum product yield was obtained with 50 mM glucose-6-phosphate and 50 μM ^{14}C-glucose. At 75 mM donor and 75 μM acceptor, inhibition was evident. The reaction was proportional to the amount of membrane protein added up to at least 195 μg (Fig. 12). This reaction, therefore, verified the localization and function of EII^{glc} in membranes.

As described previously, EI is able to catalyze the phosphoryl exchange from PEP to yield pyruvate (64). If ^{14}C-PEP is used, the amount of exchange can be determined by the amount of ^{14}C-pyruvate formed. Pyruvate is separated from PEP by reacting it with phenylhydrazine to form its osazone derivative which is water insoluble and readily separated from PEP by partitioning in ethyl acetate.

The observation that membranes alone could catalyze a PTS reaction indicated that EI and HPr are either trapped within membrane vesicles

Table 17. Glucose-glucose-6-phosphate exchange reaction (transphosphorylation) by mutanolysin-purified membranes[a] of S. mutans GS5 as a function of reactant concentrations.

^{14}C-glucose (μM)	12.5	25	50	75	100
Glucose-6-phosphate (mM)	12.5	25	50	75	100
^{14}C-Glucose-6-phosphate (nmoles formed)[b]	0.18	0.64	1.32	0.90	0.74

[a]Membranes were prepared from glucose-grown cells using mutanolysin as described in Methods.

[b]Membranes, 168 μg, were assayed in the presence of 50 mM PB, pH 6.0, 2.5 mM $MgCl_2$, 2.5 mM 2-mercaptoethanol, 10 mM NaF, and various concentrations of D-[^{14}C(U)]-glucose (4 μCi/μmole) and glucose-6-phosphate in a total volume of 200 μl. The reaction was held at 37 C for 30 min and then diluted with cold H_2O. This was held on ice until the products were separated from reactant by anion exchange chromatography. Glucose-6-phosphate was eluted with 1 M $LiCl_2$ and the eluate was counted in a liquid scintillation counter. The concentration of product formed was calculated from the measured cpm as described in Methods.

Fig. 12. Glucose-glucose-6-phosphate transphosphorylation by cell-free membranes. Cell-free membranes, prepared by using mutanolysin on cells grown in defined medium plus 20 mM glucose (see Methods), were assayed to demonstrate glucose-6-phosphate transphosphorylation. Reaction mixtures containing 70 mM PB, pH 6.0, 3.5 mM $MgCl_2$, 3.5 mM 2-mercaptoethanol, 50 mM glucose-6-phosphate, 50 μM D-[^{14}C(U)]-glucose (4 μCi/μmole) and the several membrane concentrations (total volume, 200 μl) were incubated at 37 C for 30 min. Labelled glucose-6-phosphate was separated from reactants by anion exchange chromtography (see Methods and Table 17) and measured to deduce dpm which are expressed as nmoles/200 μl.

GLUCOSE-6-PHOSPHATE (nmoles / 200 µl)
2.8
2.4
2.0
1.6
1.2
.8
.4
0
100
200
CELL-FREE MEMBRANES (µg)

or are associated with the membrane. As is evident (Fig. 13) membranes are indeed able to catalyze this exchange reaction indicating that they contain EI. Furthermore, this reaction is quite easily achieved by these preparations, being detected with as little as 2 μg of membrane protein.

Since EI is generally considered to be a soluble enzyme, it was of interest to determine if it normally is located in the cytoplasmic fraction of S. mutans. Table 18 outlines the distribution of EI during the fractionation procedure. As can be seen, 65% of the activity is associated with the soluble fractions; however, 35% is left in the membrane. Further attempts to wash the EI from the membrane with 100 mM phosphate buffer were unsuccessful. In addition, protein could not be detected in these additional washes. The small amount of activity in the second wash most likely represents EI present in the interstitial spaces of the membrane preparations. If closed membrane vesicles impermeable to cytoplasmic proteins are being formed, then trapped EI would not be expected to be eluted from such vesicles.

Membranes prepared from glucose phosphotransferase negative mutants vs. membranes prepared from wild-type cells. For this study cells of mutants and the wild-type strain were grown to mid-log phase in TYE plus mannitol. Membranes were prepared using M1 according to the description in the Methods section. Two PEP-dependent PTS reactions involving glucose and fructose were examined. The results of a typical experiment are outlined in Table 19. Of the three mutants studied, only one showed the ability to phosphorylate glucose using PEP as a phosphoryl donor. This was 8B, a mutant of the group II category. Interestingly, this showed 10% of wild-type levels which closely correlated with the activity shown by whole cells (13%, Table 11).

Fig. 13. Phosphoenolpyruvate-dependent phosphotransferase component EI activity in cell-free membranes. To demonstrate EI activity in cell-free membranes, cells were grown in defined medium plus 20 mM glucose and subjected to the mutanolysin procedure (see Methods). Reaction mixtures containing 40 mM Tris-HCl buffer, pH 7.5, 8 mM $MgCl_2$, 10 mM NaF, 2 mM pyruvate, 0.2 mM phosphoenol [1-^{14}C]-pyruvic acid (10.6 μCi/μmole) and the several membrane concentrations (total volume, 100 μl) were incubated at 37 C for 60 min. Labelled pyruvate formed during the reaction was separated from reactants by osazone formation as described in Methods. Dpm of product was determined and is expressed as pmoles/600 μl. . In a separate experiment, the variation of the assay was investigated by using eight identical samples containing 9.5 μg membrane protein in each tube. The average pmoles was 425.7/600 μl ± 22.5. This is lower than the results presented on this graph; the most probable reason for this discrepancy is EI decay (see Text).

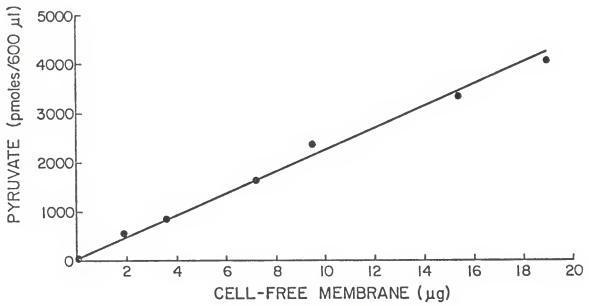

PYRUVATE (pmoles/600 µl)
5000
4000
3000
2000
1000
0
2
4
6
8
10
12
14
16
18
20
CELL-FREE MEMBRANE (µg)

Table 18. Pyruvate-phosphoenolpyruvate exchange reaction as a probe for Enzyme I: Distribution of activity in cell-free extracts[a] of S. mutans GS5.

Cell fraction	nmoles ^{14}C-Pyr/ml cell extract[e]	Total activity	% total
Cytoplasm[b] + wash 1[c]	0.06	1.500	48
Wash 2[c]	0.18	0.54	17
Membranes[d]	0.44	1.10	35

[a]Cell-free extracts were prepared by mutanolysin-induced lysis according to the procedure described in Methods.

[b]Cytoplasm refers to the supernatant obtained from the first centrifugation of lysed cells at 30,900 x g for 60 min.

[c]Wash 1 and 2 refer to wash supernatants obtained at 30,900 x g for 60 min from the first and second centrifugation of washed pellets after cell lysis. These washes were conducted using 100 mM PB, pH 7.0, containing 5 mM $MgCl_2$ plus 5 mM 2-mercaptoethanol (see Methods).

[d]Membranes refers to the final pellet obtained after the above centrifugation and removal of whole cells.

[e]The reaction mixture contained a final volume of 100 µl and consisted of 40 mM tris-HCl, pH 7.5, 8 mM $MgCl_2$, 10 mM NaF, 2 mM pyruvate, 0.2 mM phosphoenol[1-^{14}C]-pyruvic acid (10.6 µCi/µmole), and cell extracts. The extracts varied in the following manner: cytoplasm plus wash 1: 2.15 and 4.29 µg protein; wash 2: 92 µg protein; and membrane: 2-20 µg protein. Where more than one amount was used, nmoles/ml of extract/600 µl counted is expressed as the mean.

The reactants minus PEP were preincubated at 37 C and the reaction was initiated by the addition of this compound. After 60 min at this temperature, the reaction was terminated by the addition of phenylhydrazine and ^{14}C-pyruvate was detected as its osazone derivative (see Methods).

Table 19. The relative glucose and fructose phosphotransferase activities in mutanolysin-prepared membranes of mutant and wild-type strains of S. mutans GS5.[a]

	Glc PTS[b]		Frc PTS[b]	
Strain	pmoles glucose-6-phosphate/μg membrane protein	% Wild-type	pmoles fructose-6-phosphate/μg membrane protein	% Wild-type
Wild-type	12.2	100	12.50	100
3A	0	0	0.56	4.5
4B	0	0	0.57	4.6
8B	1.2	10	0.47	3.9

[a]Membranes were prepared from cells grown in TYE supplemented with 20 mM mannitol. The procedure as described in Methods was followed.

[b]Membranes, 90-180 μg protein, were incubated with 85 mM PB, pH 7.0, 4 mM $MgCl_2$, 4 mM 2-mercaptoethanol, 10 mM PEP, 10 mM NaF, and 125 μM D-[^{14}C(U)]-glucose (4 μCi/μmole) or 100 μM D-fructose containing 1 μM D-[^{14}C(U)]-fructose (359 μCi/μmole). The reactants minus the membranes were incubated at 37 C and the reaction was initiated by the addition of the membranes. After 30 min at 37 C, and the reactants were diluted with cold 1% sugar (homologous) and filtered through a DE-81 filter.

The filter was counted in a liquid scintillation counter and the pmoles of derivatized product formed was determined from the number of counts remaining on the filter after extensive washing. To insure linearity, at least two membrane amounts were used and results are expressed as a mean specific activity.

Frc PTS activity deviated from that seen in whole cells (Table 19). With whole cells, this activity ranged from 175% (3A) to 46% (8B) wild-type activity (Tables 10 and 11). However, membranes prepared from these cells showed significantly decreased activity when compared to decryptified wild-type cells. Possible explanations for this result are that the frc PTS is labile and/or there are stereochemical rearrangements of this system during membrane preparation.

It was of interest to biochemically define the site of lesion in these mutants. Since in the bacteria studied to date the PEP-dependent PT-systems involve up to four distinct proteins, a series of experiments was undertaken to more closely determine the affected protein(s). It should be reiterated that very little detail of the PT-systems in the lactic acid bacteria is known. As detailed in the Introduction, the model systems are based on studies of E. coli and S. aureus. In these organisms sugar specificity lies in the EII and EIII components (21). It was assumed that in this organism a similar situation occurred. Therefore, the transphosphorylation reaction was used to study the putative EII in these mutant strains vs. the wild-type. The data outlined in Table 20 demonstrate that this activity was not evident in either of the mutant strains examined, including the leaky strain 8B. A second set of membrane preparations yielded corroborating results. As stated previously, this assay is specific for the transport protein EII (59). It was therefore concluded that the membrane-bound glucose carrier protein(s) had been irreversibly altered.

In order to understand the physiology of these mutants in more detail, EI was assayed in both the cytoplasm and the membrane fractions.

Table 20. Transphosphorylation[a] as a measure of Enzyme II in mutanolysin-prepared membranes[b] of wild-type and mutant strains of S. mutans GS5.

Strain	pmoles ^{14}C-glucose-6-phosphate formed/µg membrane protein	% Wild-type
Wild-type	3.3[c]	100
4B	0	0
8B	0	0

[a]Transphosphorylation was carried out in an incubation mixture of 70 mM PB, pH 7.0, 3.5 mM $MgCl_2$, 3.5 mM 2-mercaptoethanol, 10 mM NaF, 50 mM glucose-6-phosphate, 50 µM D-[^{14}C(U)]-glucose (4 µCi/µmole) and membranes. To insure linearity two amounts of membrane protein were used, the lower ranged from 180-240 µg/200 µl (total reaction volume) and the upper ranged from 455-475 µg/200 µl. In the case of 4B, the amount of membranes obtained was limited; therefore, only the upper amount was used. The reactants were incubated for 30 min at 37 C and then passed through an ion-exchange column (see Methods). After extensive washing with H_2O the glucose-6-phosphate was eluted using 5 ml of a 1 M $LiCl_2$ solution. The eluate was counted in a liquid scintillation counter and the pmoles of product was calculated from the known specific activity.

[b]Membranes were prepared by mutanolysin-induced lysis (see Methods) of cells grown to log phase in TYE with 20 mM mannitol.

[c]In a separate experiment five identical samples were assayed, each containing 126 µg membrane protein. The mean amount of ^{14}C-glucose-6-phosphate formed/µg protein was 4.3 ± 0.9.

The results using two separate membrane preparations are given in Table 21. There is considerable variation between experiments, however it is apparent that the EI levels approach wild-type when both experiments are considered. The one striking difference between wild-type and mutant strains is the distribution of this enzyme. In wild-type, the membrane fraction contains 50-75% of the activity. This differs somewhat from membranes prepared from glucose-grown cells in that membranes prepared from the latter cells contained 35% of the EI activity. However, both these results differ significantly from those obtained when extracts from mutant strains are examined. It is evident that only a small fraction, 3-13% of the total EI activity is detected in the membrane fraction; the majority of it separates with the cytoplasmic constituents.

The reduced amount of frc PTS activity in membranes when compared to whole cells may be a reflection of a limiting amount of EI or of a defect in EI-binding to the mutant membranes. An attempt was made to reconstitute the membranes with cytoplasm; however, this was unsuccessful. One major problem was the decay of EI in the soluble fraction. The lability of cytoplasmic EI was not studied systematically; however, it has been observed throughout these studies. For instance in one study the level of activity of the PEP-pyruvate exchange reaction decreased by 51% and 86% in 2 and 7 days, respectively. The EI from other organisms has been shown to be labile in purified form but stable to freeze-thawing when associated with membranes (77). The same assay (PEP-pyruvate exchange reaction) using membranes of S. mutans showed only a 27% decrease in 5 days with repeated freeze-thawing. A second reason for the failure of the reconstitution experiments may be due to the impermeability of membrane vesicles to large molecular weight compounds.

Table 21. The distribution of Enzyme I as determined by the phosphoryl exchange reaction between pyruvate and phosphoenolpyruvate in cell-free extracts[a] of wild-type and mutant strains of S. mutans GS5.

	Experiment 1		Experiment 2	
	pmoles ^{14}C-pyruvate/µg protein/600 µl	% Total EI activity found in cell fractions	pmoles ^{14}C-pyruvate/µg protein/600 µl	% Total EI activity found in cell fractions
Wild-type				
Membrane[b]	48.6	52	276.0	77
Cytoplasm[c]	44.0	48	82.3	23
Total	92.6	-	358.3	
3A				
Membrane	17.0	13	12.6	6
Cytoplasm	117.0	87	212.9	94
Total	134.0	-	225.5	
4B				
Membrane	21.3	5	9.1	3
Cytoplasm	379.0	95	283.2	97
Total	400.3	-	292.3	
8B				
Membrane	21.5	10.0	23.1	13
Cytoplasm	200.0	90.0	153.0	87
Total	221.5	-	176.1	

[a]Cell-free extracts were prepared by mutanolysin-induced lysis of cells grown to log phase in TYE supplemented with 20 mM mannitol. The procedure followed is as outlined in Methods.

[b]Cell membranes were incubated in 25 mM tris-HCl, pH 7.5, 12 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 2 mM pyruvate, and 0.2 mM phosphenol [1-^{14}C]-pyruvic acid (10.6 µCi/µmole). Since membranes were suspended in PB, pH 7.0, this buffer was present at a concentration of 40 mM. A linear range of 5-45 µg of protein was assayed for each strain listed. The total assay volume was 100 µl.

[c]Soluble extracts were in tris-HCl, pH 6.8. Therefore, the following conditions were employed: 12 mM tris-HCl, pH 6.8, 10 mM $MgCl_2$, 3 mM 2-mercaptoethanol, 10 mM NaF, 2 mM pyruvate, and 0.2 mM phosphoenol[1-^{14}C]-pyruvic acid. Amounts of extracts (2-12 µg protein) within a linear range of activity were used. The total assay volume was 100 µl.

In b and c, the reaction mixture was incubated for 60 min at 37C and ^{14}C-pyruvate was detected as its osazone derivative (see Methods).

Regulation of the lactose phosphotransferase system by monosaccharides. If non-induced cells are grown in a medium containing glucose and lactose, a biphasic growth pattern is observed. This can be seen in Fig. 14. The first phase of growth coincides with the uptake of glucose whereas the second phase of growth coincides with the uptake of lactose. These two phases of growth are separated by a 60-min lag period. Glucose is completely exhausted from the medium before lactose is taken up by the cells, and lactose uptake is preceded by a typical diauxic lag period. The lag period usually is indicative of the induction of enzymes required for uptake of the second sugar. This appears to be the case in these cells. The induction of the lac PTS, as measured using the chromogenic analogue ONPG, is concomitant with the second phase of growth and, furthermore, is not evident until the lag phase is completed. Its specific activity then increases coincident with the second phase of growth. Phospho-β-galactosidase, on the other hand, appears during the first phase of growth. This enzyme can be detected, albeit at low levels, in glucose-grown cells (data not shown). Its synthesis appears to precede the synthesis of the lac PTS (Fig. 5). The possibility exists that one of its products, galactose-6-phosphate, is an inducer of the lac PTS as has been found in S. aureus (43).

It appears that the presence of glucose is able to prevent induction of the lac PTS in S. mutans. However, it does not appear to regulate lactose metabolism once the lactose dissimilating enzymes are present. This can be seen from the data listed in Table 22 where lactose-grown cells were grown in a glucose-lactose mixture. Even though glucose uptake seems to precede lactose uptake, once log phase growth commences

Fig. 14. Diauxic growth patterns by S. mutans GS5 using glucose and lactose substrates. Log-phase cells grown in defined medium plus 5 mM glucose were harvested, washed with sterile H_2O (2 ml), and resuspended in 198 ml defined medium plus 5 mM glucose and 5 mM lactose. At specified times, 10-ml aliquots were removed for absorbancy reading on the Klett (▲). The cells were centrifuged and washed once with 10 ml of PB, pH 7.0, plus 5 mM $MgCl_2$. A_{600} measurements were made to determine dry weights of each cell preparation. Cells then were resuspended in 1 ml buffer and decryptified. Assays for the lac PTS and phospho-β-galactosidase (inset) were done using ONPG + PEP (●) and ONPG-6-P (●) respectively. The volume of cells used for these assays was 50 μl. The spent medium from the original centrifugation was assayed for glucose (■) and lactose (●) as described in Methods.

SUGAR UPTAKE µg/ml
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
KLETT UNITS
160
140
120
100
80
60
40
20
0
0
200
300
400
500
600
TIME (min)
GROWTH
GLUCOSE
LACTOSE
ONP FORMED(µmoles)/MIN/µg CELLS/ml
× 10⁻⁵ ONPG-6-P
or × 10⁻⁶ ONPG+PEP
60
40
20
0
200
400
600
MINUTES
ONPG-6-P
ONPG+PEP

Table 22. The growth and sugar uptake by cells of S. mutans GS5 induced for lactose dissimilation in medium supplemented with lactose and glucose.[a]

Minutes	Klett units	Disappearance of sugar from medium	
		Lactose[b]	Glucose[b]
0	7	0	0
180	15	0	1.0
240	22	0	1.4
300	38	1.2	1.9
345	52	0.5	1.5
370	66	1.8	2.0
390	83	2.5	2.5
430	115	3.9	3.6
450	137	4.8	1.7
540	175	6.5	3.3
575	185	6.6	4.1
595	186	ND[c]	ND

[a]Cells were grown in 10 ml defined medium supplemented with 5.0 mM lactose (to log phase). This culture was transferred to 100 ml defined medium containing 5 mM lactose plus 5 mM glucose. At specified times, 5 ml aliquots were removed to measure Klett units. Cells were removed from this aliquot by centrifugation and the lactose and glucose concentration in the spent medium was determined according to the methodology described under Methods.

[b]The concentration determined at each point was subtracted from the starting concentration and this difference, in mM, is the expressed result.

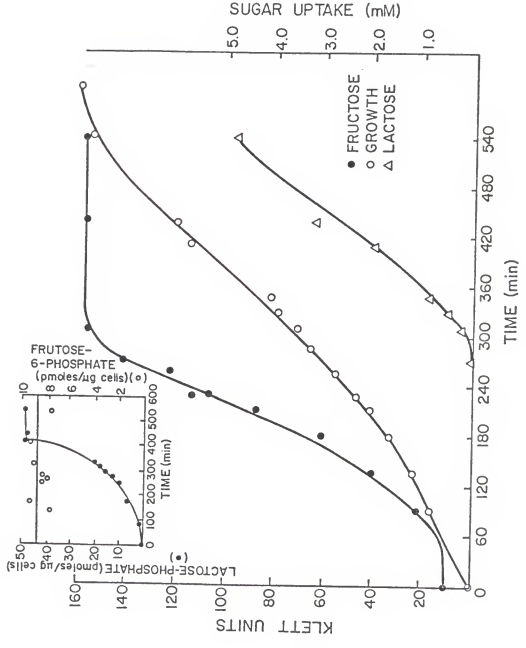
[c]Not determined.

both are utilized in parallel. The slightly higher than expected concentration of lactose found in the medium most likely reflects carry-over from the inoculum.

One interesting point is the apparent expulsion of glucose from cells at 450 min. This was a reproducible observation. Reizer and Panos (52) have shown that Streptococcus pyogenes exhibits a phenomenon they termed "inducer expulsion." When these cells are allowed to transport TMG (forming phosphorylated TMG) and are presented with a metabolizable sugar such as glucose, free TMG is expelled. However, in the case presented in Table 22 it is the metabolizable compound being excreted. A simpler explanation may be that free glucose, formed from the cleavage of lactose-phosphate to galactose-6-phosphate plus glucose, is being expelled in favor of the glucose-6-phosphate formed by the glc PTS.

Fig. 15 shows the results of growing non-induced cells in fructose and lactose. Growth is not apparently biphasic. However, a semi-log plot of the data reveals two growth rates; the first is equal to a generation time of 82 min and is commensurate with fructose uptake (0-310 min; Fig. 15) while the second rate is equal to a generation time of 225 min and commences with lactose uptake (310 min; Fig. 15). Lactose uptake does not begin until the exhaustion of fructose from the medium. However, as is demonstrated in Fig. 15, a diauxic induction period is not evident. Indeed the induction of the lac PTS proceeds parallel with growth (inset Fig. 15). A semi-log plot of the data confirms this conclusion. The frc PTS does not vary since it has been optimized by prior growth in fructose.

Fig. 15. Growth patterns displayed by S. mutans GS5 in fructose plus lactose. Log phase cells, grown in defined medium plus 5.0 mM fructose (10 ml) were transferred directly to 90 ml defined medium supplemented with 5 mM fructose plus 5 mM lactose. At specified times aliquots were removed for absorbancy readings on the Klett (○) and cells were removed by centrifugation. Spent medium samples were assayed for fructose (●) and lactose (△) contents as described in Methods. Cells were washed and dry weights determined. To measure for frc PTS activities, washed cells were decryptified and suspended in 60 mM PB, pH 7.0, 3.0 mM $MgCl_2$, 10 mM PEP, 10 mM NaF, and 1.5 μM D-[^{14}C(U)]-fructose (359 μCi/μmole) to a final volume of 200 μl. The cell concentration was 18-20 μg/200 μl. The reaction proceeded for 30 min at 37 C, was stopped with excess cold fructose and filtered through a DE-81 filter. Labelled fructose-6-phosphate (○) is expressed as pmoles/μg cells (inset). The lac PTS was determined in a similar manner except the reaction mixture (200 μl) contained 210 μM[^{14}C(U)]-lactose (0.97 μCi/μmole) and the reaction was terminated in cold lactose. Labelled phosphorylated derivatives (●) are collectively expressed as μmoles/μg cells/200 μl (inset).


SUGAR UPTAKE (mM)
5.0
4.0
3.0
2.0
1.0
FRUCTOSE
GROWTH
LACTOSE
TIME (min)
0
60
120
180
240
300
360
420
480
540
KLETT UNITS
20
40
60
80
100
120
140
160
LACTOSE-PHOSPHATE (pmoles/µg cells)
(•)
50
40
30
20
10
0
100
200
300
400
500
600
TIME (min)
FRUTOSE-6-PHOSPHATE (pmoles/µg cells)(o)
10
8
6
4
2

In order to compare the regulation of the lac PTS of the mutant strains with the wild-type strain, cells were pre-grown in TYE plus 20 mM mannitol for 16 h. As can be seen in Table 7, cells grown under these conditions do not exhibit a lac PTS. A 5% inoculum of such cultures was used to inoculate a Klett tube containing TYE supplemented with 5 mM lactose and 5 mM glucose. The tubes were incubated at 37 C in a candle jar and at various times, the absorbancies were measured in a Klett-Summerson photometer. The results of a growth curve using the leaky strain 8B and wild-type cells are shown in Fig. 16. With wild-type cells, as expected, two phases of growth are separated by a lag phase. Transformation of these data via a semi-log plot (not shown) revealed two growth rates. The first phase of growth showed a mean generation time of 64 min whereas the second phase corresponded to a mean generation time of 120 min. The mutant strain exhibited a uniphasic growth pattern and this was confirmed by plotting the data as a semi-log graph (not shown). Here, the mean generation time was calculated to be 71.6 min. Interestingly, the cell density was equal for both strains suggesting glucose was fully taken up by the mutant strain. As previously noted, strain 8B is leaky for the glc PTS. In addition, S. mutans has been shown to contain an alternative glucose transport system which plays a significant physiological role under high glucose conditions. PTS negative mutants of S. mutans have been shown to transport glucose (18).

Fig. 17 demonstrates the differential onset of ^{14}C-lactose uptake by wild-type cells as compared to the mutant strain 3A in the presence of glucose. This mutant strain appeared not to have a functional glc PTS (Tables 10 and 19). (Interestingly this strain did show delayed

Fig. 17. Lactose uptake by S. mutans GS5 (wild-type) and a glucose phosphotransferase negative mutant as a function of cell density. Cells (wild-type or glc PTS^- mutant strain 3A) were grown for 16 h in TYE plus 20 mM mannitol. After harvesting, washing, and concentrating two-fold, a 10% inoculum was used to initiate growth in 10 ml TYE supplemented with 5 mM glucose plus 4.9 mM lactose (4.7 mM [^{12}C]-lactose plus [^{14}C]-lactose: 0.97 μCi/μmole). Cultures were incubated at 37 C in a candle jar. At specified absorbancies as read on the Klett, aliquots (0.1 ml) were removed to measure counts per minute (cpm) taken up by cells (wild-type △, 3A ●).

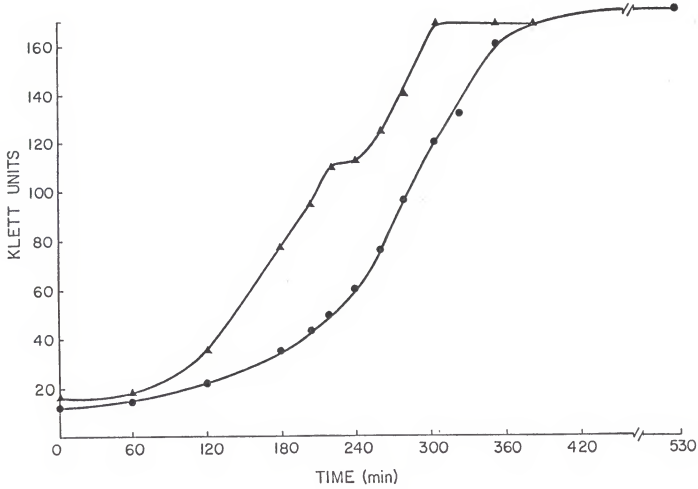
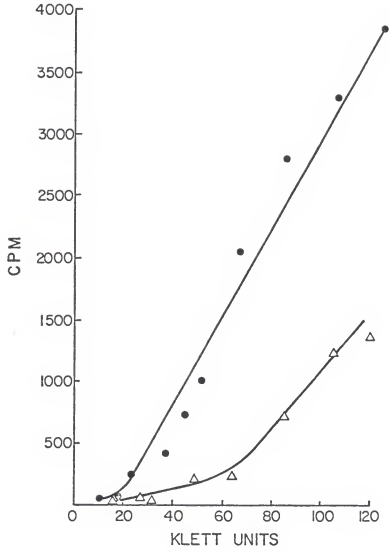

KLETT UNITS
160
140
120
100
80
60
40
20
0
60
120
180
240
300
360
420
530
TIME (min)

Fig. 16. Growth of S. mutans GS5 (wild-type) and a glucose phosphotransferase negative mutant in lactose plus glucose. Cells (wild-type or glc PTS^- mutant strain 8B) were grown for 16 h in TYE plus 20 mM mannitol. Five percent inocula were used for growth in TYE supplemented with 5 mM glucose plus 5 mM lactose. Cultures were incubated at 37 C in a candle jar and absorbancies (wild-type ▲ , 8B ●) were measured using a Klett.


4000
3500
3000
2500
2000
1500
1000
500
CPM
0
20
40
60
80
100
120
KLETT UNITS

but sparse growth on TYE agar plates supplemented with glucosamine.) The experiment in Fig. 17 was done by transferring cells grown in TYE plus 20 mM mannitol to Klett tubes containing TYE plus 5.0 mM glucose and 4.9 mM lactose of which 0.21 mM was the radioisotope. At specified times, an absorbancy reading was obtained and 100 µl were removed for filtration through a 0.2 µ membrane filter (Gelman Sciences, Inc., Ann Arbor, MI.). These filters were washed extensively with H_2O and transferred to 5 ml Aquasol for counting. Duplicate counts were obtained. (A second experiment using centrifugation yielded similar results to those shown here.)

Lactose uptake, as measured by counts incorporated in cells (Fig. 17), correlated with growth of the mutant strain (data not shown); that is, even in the presence of glucose, the mutant's primary growth substrate is lactose. On the other hand, even though lactose internalization is evident at an early stage of growth in wild-type cells, it is significantly depressed and this repression is not relieved until the second phase of growth as would be predicted by data shown in Fig. 14. Thus, there are two phases of lactose uptake. The first phase of uptake is at a low rate, 5.2 cpm/Klett unit, and may be explained as the internalization of inducer. The second rate, 19.4 cpm/Klett unit, most likely represents the utilization of lactose as a growth substrate. These rates can be compared to that observed for 3A which was 40 cpm/Klett unit. Rates were obtained by linear regression analysis and these data gave correlation coefficients of .985 for 3A and .952 and .872 for the first and second phases observed for wild-type.

As in other growth studies (Fig. 16), two growth rates were obtained with wild-type culture (mean generation times 95 and 165 min, respectively). However, a single growth rate with a mean generation time of 109 min was observed for strain 3A. The maximum growth obtained for this strain was less than with wild-type. As measured in Klett units, the maximum absorbancy was 140 vs. 170 for 3A and wild-type GS5, respectively. The cell densities achieved by the respective strains reflect the amount of glucose left in the spent media. In a separate experiment where glucose was measured, wild-type GS5 exhausted the glucose (5.0 mM) from the medium whereas 2.2 mM remained after the cessation of growth of 3A. Again, uptake of glucose by mutants may be explained by the observation of Hamilton and St. Martin (18) that the alternate glucose transport system is a low affinity system.

CONCLUSIONS

Previous studies on the cariogenic organism, S. mutans have been concerned mainly with the ecology of this organism, with emphasis on the initiation of pathogenesis. Not emphasized in the literature is the relationship between the physiology of this microbe and pathogenesis. In the oral cavity, S. mutans adheres to the tooth surface where it establishes a focus of infection through various mechanisms. These mechanisms of adherence have been studied extensively (13,14) and will not be discussed here. Cariogenicity is actually caused by metabolic events occurring subsequent to the initial attachment of the bacteria to dental enamel. It is the production of lactic acid from the fermentation of a select group of sugars that is responsible for the degradataion of the dental structure (13,14). Knowledge concerning the physiology of this streptococcus, however, is minimal. It is known that this organism grows on a limited number of sugar substrates and that these substrates are transported by PEP-dependent phosphotransferase systems (Table 1). This mode of transport can be viewed as the first step in fermentation since the sugar becomes modified to the first intermediate in its dissimilatory pathway during passage through the membrane.

This dissertation describes the glc PTS in detail since this sugar very likely represents the major carbon source of S. mutans. Since basic knowledge concerning transport of this monosaccharide

is limited, the focus of this project was general in nature. This study deals with three interrelated areas and in each case attempts to obtain a basis for further study. The objectives of these areas were thus: (1) to obtain a general description of the glc PTS in whole cells (e.g., its substrates, under what growth conditions it is present, etc.); (2) to obtain a more detailed description of the glc PTS by using cell-free extracts (e.g., the existence of soluble vs. membrane factors); and (3) to definitively ascribe a regulatory function of the glc PTS over the transport of other PTS substrates.

General studies on the glucose phosphotransferase system. The methodology used for these studies allowed the study of phosphorylation rather than actual transport. The reason for studying phosphorylation was the convenience of this approach and the elimination of competing alternate systems (i.e., those driven by the proton motive force or by ATP) when decryptified cells were used. Much evidence has been accumulated to strongly suggest that group translocation (i.e., phosphoryl transfer) and transport are tightly coupled processes in intact cells. These include the inability of EI- and HPr-negative mutants to accumulate sugars even though the permease (EII) is present. Sugars are not transported even by means of a facilitated diffusion mechanism in such mutants (21,49,78). More direct evidence comes from the work of Kundig and Roseman (30) showing that the isolated PTS components require a membrane factor to ultimately transfer the phosphoryl group to a sugar. Parallel work in the laboratory of Hengstenberg (26) demonstrated the tight coupling of phosphorylation and transport in S. aureus. Thus, by defining PEP-mediated phosphorylation, one is also describing the underlying mechanism for transport.

Using the two glc PT-systems of E. coli as models, initial studies were concerned with defining the nature of the glucose system(s) in S. mutans. Early in the study, it was observed (Tables 7 and 8) that S. mutans GS5 was capable to transporting 2-DG but not α-MG. In addition, glucose, mannose, and 2-DG transport systems appeared to be constitutive. This indicated that a system somewhat analogous to the low affinity system of E. coli exists in this organism. But in E. coli, this system transports glucose, mannose, 2-DG, and fructose (21,30). Upon further investigation, S. mutans appeared to diverge from the above model. Results of competition experiments (Table 9) showed that glucose and fructose do not share receptor sites on common PTS proteins. However, the results of these experiments supported the conclusion that a glc/man PTS was present in this organism and that this system also utilizes 2-DG as a substrate. A puzzling observation was the competition between fructose and mannose. This observation may be the result of one or a combination of several factors: (1) the existence of an ATP-dependent manno-fructokinase, (2) the affinity of the frc PTS for mannose, or (3) the affinity of a "mannose site" on the glc/man PTS for fructose.

Kinetic studies indicate that the affinity of the glc/man PTS is: glc > man > 2-DG. The relative growth rates of these cells in the various carbon sources reflect these relative affinities. Thus, the K_m value for glucose and mannose are 64 μM and 90 μM and the mean generation times are 75 and 290 min, respectively. An observation relevant to this discussion is the extent of inhibition each sugar exhibited for the heterologous phosphorylating reaction (Table 9). That is,

glucose is a more effective competitor of mannose phosphorylation (96% inhibition) than mannose is of glucose phosphorylation (74% inhibition). Since growth in mannose is so poor, it would seem that factors in addition to the relative affinity of the PTS for mannose must be responsible. Interestingly the V_{max} for these two sugars are similar (.366 nmoles/min/ml for glucose vs. .300 nmoles/min/ml for mannose). This further indicates a factor beyond transport is responsible for the limited growth of S. mutans in mannose.

Results obtained using mutants selected specifically for glc PTS negativity confirm and extend the conclusions presented thus far: (1) in this organism there are distinct PT-systems for the transport of glucose and mannose vs. fructose, and (2) glucose is a more efficient substrate for the glc/man PTS than is mannose. Tables 10-12 demonstrate these points. Apparently tight, as well as moderately leaky, glc PTS negative mutants were unable to phosphorylate mannose (Tables 10 and 11). Mutants with an increased capacity to phosphorylate glucose (30%; Table 12) showed moderate mannose phosphorylation. This pattern again suggested that the glc/man PTS phosphorylated (transported) glucose more efficiently than mannose and that a minimum amount of glc PTS activity (30%) must be present before mannose phosphorylation is evident. Fructose phosphorylation again appears to be an independent reaction in the selected mutants described here.

One of the more relevant observations in terms of the overall physiology of this organism is the determination of a separate frc PTS. This organism is characterized by its possession of glucosyltransferase on its cell surface. This enzyme synthesizes capsular dextran from

sucrose providing the cells with the capability of adhering to enamel surfaces. In order to obtain the glucose necessary for dextran polymerization, sucrose is first split by this enzyme with the formation of free fructose (13,14). Fructose is then taken up by the frc PTS and metabolized to lactic acid. Thus, fructose plays an important role in the physiology and pathogenesis of this organism.

Collectively, these data indicate that the glc PTS resembles the low affinity glc PTS of E. coli. This is in agreement with results obtained in other laboratories working on S. lactis (86) and S. faecalis (27). Unfortunately, the work in these laboratories and the studies presented here are inconclusive. To demonstrate the molecular nature of the glc PTS in S. mutans, it is necessary to isolate and describe the individual PTS proteins as was done for E. coli (30,31; see Introduction) and S. aureus (26,76,77; see Introduction). The next section describes an attempt to set a foundation to begin the molecular dissection of the glc PTS in this species.

Studies on the glucose phosphotransferase system using cell-free extracts. Establishing the various parameters for glc PTS activity in whole cells allowed this work to progress to the level of the cell-free system. The method of choice for preparing such a system was that described by Siegel et al. (75) using the muralytic enzyme, mutanolysin (M1). Before proceeding with any further discussion concerning the use of cell-free extracts, it would be helpful to examine the advantages and limitations involved in the use of this methodology.

The advantages of using M1-prepared membranes, i.e., membranes prepared from osmotically-lysed cells vs. membranes prepared from

mechanically-broken cells were: (1) the higher specific activity in terms of the PTS, and (2) the apparent reflection of these membranes of the physiology of whole cells, at least as measured by the various PT-systems. This latter conclusion was based on the comparison of the PTS-induction patterns (Table 15) and the results of competition experiments (Table 16) using membranes with data obtained using permeabilized cells. Membranes prepared from glucose-grown cells were able to phosphorylate glucose, mannose, and fructose; thereby reflecting the constitutive nature of these PT-systems found in the cells from which they were derived. The pattern of competition using radiolabelled sugars, as elucidated in whole cells, was reproduced using membrane preparations leading to the conclusion that a glc/man PTS, which also phosphorylated 2-DG, and a separate frc PTS were present in these membranes.

A third advantage of using M1-prepared membranes is the resulting clean separation of cell-wall and membrane material one is able to attain with this procedure (75). Presumably, this yields a purer product than that obtained with mechanically broken cells; Siegel et al. (75) demonstrated this to be true with respect to contaminating cell wall.

A major limitation of this technique can best be understood by discussing the data presented in Tables 13 and 14. Table 13 demonstrates the fractionation of PTS activity during the separation of the cellular extract into the "cytoplasmic" and "membrane" fractions. It is evident that all the activity is retained in the membrane fraction. Theoretically "purified" membranes should not be active since they should lack the soluble proteins. However, the apparent activity found in these membrane preparations brings into question the absolute purity of these membranes.

As stated, it has been demonstrated that membranes prepared by M1 are free of cell wall; however, results presented in Table 14 indicate cytoplasmic contamination, albeit at low levels. These results demonstrate that in the absence of PEP and NaF phosphorylation of glucose still occurs; thus, indicating the presence of an energy reserve in the form of glycolytic intermediates. The inclusion of NaF inhibits this background suggesting the presence of at least one glycolytic enzyme, enolase, since NaF specifically inhibits that enzyme. These data may be interpreted to mean that membranes containing cytoplasmic constituents may be present in these preparations. In order to pursue some of the more intriguing results obtained during the course of these studies, it would be necessary to purify these membranes further, if possible, perhaps by sucrose-density gradient centrifugation or by hydrophobic chromatography.

A second disadvantage is the limitation on quantitation inherent in the methodology. The conclusion that M1-prepared membranes were physiologically similar to the cells from which they were prepared was a qualitative one. It is evident from Table 15 that an appreciable loss of activity occurred during the isolation procedure. Quantification of this loss was not made due to the inability to accurately measure the amount of membrane protein. For instance, the amount of M1 contaminating the cell fractions was unknown; therefore, the amounts of membrane protein employed in these studies reflected relative rather than absolute amounts. One can only speculate as to the reasons for the apparent loss of activity. For example, it may be stereochemical rearrangement of membrane proteins within the lipid matrix, a denaturation of the proteins, or a loss of "soluble" PTS proteins such that HPr and EI are in limiting quantities.

One question yet to be answered is the extent of permeation of these membranes by large protein molecules. One assumes that these membranes form closed vesicles in aqueous suspensions based on thermodynamic considerations. However, the extent of leakiness of such "vesicles" remains to be demonstrated. This is a question which repeatedly presented itself throughout the many aspects of these studies. For example, Table 13 illustrates the failure of cytoplasm to increase the glucose phosphorylating activity of cell-free membranes. The question then can be raised as to whether this failure is due to the impermeability of these "vesicles" to high-molecular weight compounds or the presence of saturating amounts of EI and HPr in such preparations. It is obvious that answering this question would shed some light on the molecular interactions of the individual PTS components in this species.

The major advantage of a cell-free system is that it allows the study of the individual PTS proteins. The two proteins examined were the EI and EII^{glc}. The reason these were chosen for study is that assays have been described for each (56,64) that allow direct measurement, thereby obviating the need for the isolation and purification of these proteins.

S. mutans does appear to have a typical EII^{glc} in that it not only functions in transport utilizing PEP as a donor but also catalyzes the phosphoryl exchange reaction (transphosphorylation; Table 17) between glucose and glucose-6-phosphate. It has been reported that the transphosphorylation reaction is one-tenth as active as the PEP-mediated reaction (56). For example, a typical result of 23.5 pmoles product/μg membrane protein when PEP is used is to be compared with 2.3 pmoles

product/μg membrane protein when glucose-6-phosphate is the phosphoryl donor. In both these cases products and reactants are separated by ion exchange chromatography. It may be noted that there is a discrepancy in the results when comparing those obtained by chromatography vs. those obtained by ion-exchange membrane filtration (Table 15). This is due to the greater capacity of the columns. It is for this reason that columns are used in the transphosphorylation assay (Dr. G. Jacobsen, personal communication). Since cold glucose-6-phosphate is in 1000-fold excess of ^{14}C-glucose, the cold compound occupies many of the binding sites on the filter and thus effectively competes with the ^{14}C-glucose-6-phosphate formed. This permease (EII^{glc}) is similar to that described for E. coli (59) in that the transphosphorylation reaction is subject to severe substrate inhibition (Table 17).

The exchange reaction between PEP and pyruvate allowed the identification of an EI-like protein in both the membrane and cytoplasmic fractions. The observation that membranes alone catalyzed a phosphorylation reaction with PEP as the phosphoryl donor implied the presence of such a protein; the direct demonstration of this reaction demonstrated conclusively that this normally soluble protein is contained in these cell-free membrane preparations. Membrane vesicles prepared from E. coli by osmotic lysis (Kaback vesicles) have been shown to contain low levels of EI and PEP (12). Indeed Kaback (28) used these vesicles to demonstrate PEP-dependent transport.

Results from studies of fractionation of EI activity during membrane isolation reveal that the major activity (65%) fractionates with the cytoplasm. However, a significant amount (35%) fractionated with the

membranes. Elution of EI from membranes using phosphate buffer was uncussessful. This simply may be due to an incorrect choice of buffer. Alternatively, it may be due to the trapping of EI (and presumably HPr) in closed vesicles. The trapping may be the result of non-specific events involving the closure of membranes in the presence of cytoplasm or it may be the result of a specific association of EI with membrane receptors (perhaps the EII^{glc}) if, in fact, EI (HPr) are extrinsic membrane proteins. Singer and Nicholson (79) define extrinsic membrane proteins as those which fractionate with membranes but can be removed by relatively gentle means such as changes in ionic strength or pH. However, this definition does not always allow for conclusive results.

The possibility that EI is an extrinsic membrane protein is supported by three observations. The first is the increased lability of EI observed in cytoplasmic vs. membrane preparations. The membrane association may protect EI from oxidation. The second is the significant fraction (35%) of the total EI (cytoplasm plus membrane) isolated in the membrane preparation. However, the possibility exists that much of the cytoplasmic EI content was oxidized before assaying and therefore the amount calculated was greatly underestimated. A third more interesting observation comes from the study of mutant membranes. Membranes prepared from the various classes of glc PTS negative mutants appeared to have normal levels of total cellular EI, however the amount within the membrane was greatly reduced when compared to wild-type membrane (Table 21). It is attractive to suppose that the EII^{glc} is a major receptor for EI (or HPr) and the loss of a normal permease results in the inability of EI to maintain its membrane association.

Again, it is difficult to draw definitive conclusions from data obtained using mutant membranes since complete separation of cytoplasm from membranes was not achieved. For instance in Table 21, experiment 2, 77% of the EI was detected in the membrane of wild-type cells which is a much higher amount than had been previously observed. One reason for this higher figure may be related to the distribution of protein, 47% of the total protein was found in the membrane fraction in this experiment as compared to 20% in the first experiment. This is certainly the result of cytoplasmic contamination.

The above discussion highlights the necessity for obtaining pure membranes in order to be able to draw reliable conclusions. However, these data do demonstrate the existence of an EI-like protein in this organism. Its cellular localization is a more difficult determination. One approach that may shed some light on this question is to determine the sidedness of these presumed membrane vesicles. If, for instance, 35% of the vesicles are inside-out then 35% of the extrinsic proteins (e.g., EI) would be washed from the membrane with low ionic strength buffer. Of vesicles prepared by osmotic lysis of E. coli approximately 15% are everted, whereas 95% of the vesicles prepared in the French pressure cell are everted (12). Vesicles possibly existing in M1 membrane preparations would be expected to be similar to Kaback vesicles since both are prepared by osmotic lysis. The determination of the absolute amount of everted vesicles would be quite instructive in planning further experimentation.

Regulatory functions of the glucose phosphotransferase system. In addition to transport, the glc PTS in E. coli plays a role in regulation

of other transport systems (see Introduction). One of the main objectives of this study was to assign a regulatory role specifically to the glc PTS as has been done for this enteric microbe.

A regulatory role for the glc PTS in S. mutans has been controversial. Hamilton and Lo (16) observed a diauxie when S. mutans was grown in a combination of lactose and glucose; however, this diauxie was "leaky" in that glucose-induced repression was concentration-dependent. The growth history of the cells used in their experiments was not presented so that it is difficult to evaluate the results obtained by these authors. Thompson et al. (90) and Heller and Röschenthaler (23) have argued in favor of a competition of the various PT-systems for the common components, with certain systems being more efficient (e.g., glucose) than others and causing an inhibition of the less efficient systems (e.g., lactose). The data presented in this dissertation demonstrates the repressive effect the glc PTS has on the synthesis of the lac PTS in cells of S. mutans (Fig. 14). This regulatory role appears to be specific for the glc PTS. The frc PTS is able to inhibit the transport of lactose but not repress the synthesis of the lac PTS (Fig. 15). It is possible, therefore, that regulation may be dependent on a functional EII^{glc}.

In order to clarify the role in the regulation of sugar transport of the glc PTS in general and the EII^{glc} in particular, glc PTS^- mutants were utilized. The rationale for taking this approach was to obtain a mutant sub-strain of GS5 showing wild-type PTS activity for all PTS functions except those unique for glucose and in addition was able to transport glucose through an alternate system. This would provide a valuable

tool for the study of the hierarchy of sugar uptake. Before proceeding with a discussion of regulation, I must diverge and discuss the nature of the selected mutants to meet the above criteria.

Tables 10 and 11 outline the various PTS activities of selected clones. The clones I chose to study were based not only on these activities but also on their growth characteristics. One of the most promising mutants was 3A since not only did this strain appear to have equivalent or greater levels of the frc and mtl PT-systems when compared with wild-type strains indicating wild-type levels of EI and HPr, but 3A also appeared to have the same growth characteristics as the wild-type in TYE plus mannitol. Other mutants chosen to be examined in detail were 8B, 4B and 2A (data not presented).

All mutant strains appeared to contain EI, as measured by the PEP-pyruvate exchange reaction, at levels comparable to wild-type. When cell-free membranes of mutants were prepared, they were found to be similar in their lack of functions dependent on EII^{glc}. All but 8B lacked PEP-dependent glucose phosphorylating activity. Mutant 8B membranes showed 10% of wild-type levels of the latter activity; this result is in agreement with that obtained with whole cells (13%, Table 11). These data substantiated the conclusions reached with studies using the whole cells from which these membranes were derived, namely, that the lesion is in the sugar-specific component(s) of the glc/man PTS. Contradictory, however, were the results obtained when fructose was used as a substrate. Unlike studies with intact cells, all membranes derived from mutants showed reduced levels of PEP-dependent phosphorylation (Table 19). It is difficult to determine the reason for this discrepancy

without fully defining the biochemistry of the isolated mutants, however speculation has led to a number of possibilities. One possible reason for these results is that mutagenesis yielded fragile membranes either because of damage to the EII^{glc} or because of unspecified secondary events. In the former case one could hypothesize that the EII^{glc} plays a critical structural role; a hypothesis that has some support in light of the data collected on the distribution of EI in mutant vs. wild-type cell extracts (Table 21). The results of these studies indicate that such a structural alteration in the case of these mutants would only be apparent when the membranes are perturbed much like the situation of the ATPase of E. coli. This enzyme was found to change its orientation upon membrane isolation (69). A simpler explanation is the amount of EI may be below saturating levels in these membranes perhaps because of the damage to the EII^{glc} (as discussed in the previous section). Complementation experiments with isolated PTS components would allow the evaluation of the validity of the latter point.

The conclusion that the major lesion in these mutant cells is in the EII^{glc} was substantiated by the failure to observe a transphosphorylation reaction; a reaction which requires this membrane-bound protein complex only (59), and on the apparently normal levels of EI (and HPr) as shown by direct measurements of total cellular EI (Table 21). This does not rule out a secondary lesion in the EI or HPr proteins which prevents their specific association with the EII^{glc} to block PEP-dependent phosphorylation of this sugar. However, given the non-specificity of HPr and EI found in other genera (21), this would seem unlikely.

Glc PTS negative mutants were still able to utilize glucose, most likely through a transport mechanism driven by the proton motive force

(18), however, these cells no longer show the typical diauxic growth pattern demonstrated by wild-type cultures growing in glucose plus lactose (Fig. 16). The localization of the lesion in the EII^{glc} is the first step in elucidating a possible mechanism of regulation. In E. coli, regulatory functions of the PTS are carried out by the non-specific components (EI and HPr) and by the high-affinity glc EIII (see Introduction). A definitive determination of regulatory functions in S. mutans would require biochemical fractionation of the wild-type vs. mutant PTS proteins along the lines advanced by Kundig and Roseman (30, 31) in their studies of E. coli. It is an approach such as this that allows the definitive determination of the functionality of the individual PTS proteins. In vitro recombination of mutant and wild-type proteins also would aid in the elucidation of which functions are affected by mutagenesis.

The similarity of the well-studied regulatory role of the E. coli glc PTS to the S. mutans glc PTS extends only to the observation that glucose is the preferred sugar in non-induced cells of both species. In E. coli, regulation is complex involving distinct mechanisms (inducer exclusion vs. catabolite repression) and distinct effector molecules (cAMP). Furthermore, regulation presumably involves the PTS functioning to control non-PTS-mediated transport (e.g., glucose vs. lactose); thus, a role for EI and HPr can be ascribed. In S. mutans, regulation of one PTS over a second occurs. It should be noted here that even though the focus of recent studies in E. coli has been on the PTS control over non-PTS-mediated transport, diauxie has been observed when cells are grown on two PTS substrates as in the cases of glucose plus fructose (21)

and glucose plus sorbitol (34). Very little is known about this control mechanism and its elucidation would probably aid in our understanding of regulation in S. mutans.

Because EI and HPr function in all PTS-mediated reactions, it is difficult to envision a regulatory role for these two proteins in the case of glc PTS control over other PTS-mediated transport. However, it is plausible that a phospho-EIII^{glc}, similar to that of E. coli, exists in S. mutans and is able to inhibit heterologous permeases (EII). Thus, in the presence of glucose, phospho-EIII^{glc} is formed primarily to transport glucose and secondarily to inhibit other permeases, thereby causing inducer exclusion. Again, it would be informative to determine if a soluble EIII exists before proceeding with further experimentation. If upon fractionation and isolation of the various PTS components such a protein is found, it would then be of interest to add that protein, in a phosphorylated form, to a cell-free system derived from lactose-grown cells to determine if it inhibits lactose phosphorylation. Again, the permeability barrier may have to be overcome.

Though I favor the above model, other proposals must be considered. It has been proposed that various phosphorylated metabolites react with the several permeases, thereby causing inducer exclusion. This has been proposed for the observed diauxie in E. coli between glucose and fructose (two PTS substrates) and has been given some support since the discovery of the transphosphorylation reaction (21). A priori one would expect glucose-6-phosphate to be formed both during glucose and lactose metabolism, therefore it is difficult to imagine how this compound or any of its subsequent metabolic products could have a negative regulatory

function in lactose transport. Also, transphosphorylation involves a sugar and its homologous phosphorylated derivative (59,60), therefore the only plausible control mechanism one could postulate is a feed-back inhibition of that specific transport system.

An attractive hypothesis has been put forth by Thompson et al (90). They propose that catabolite inhibition is responsible for the preferential utilization of glucose over other PTS sugars. That is, the affinity for HPr by the glucose-specific PTS proteins is greater than by the other sugar-specific PTS proteins (e.g., lactose). If this were the case then a less stringent diauxie than that observed here (Fig. 14) would be expected. Indeed Hamilton and Lo (17) demonstrated that diauxie in the strain of S. mutans they were studying was concentration dependent. That is, as the glucose concentration was lowered, induction of the lac PTS occurred. This would be an expected observation if competition between two PT-systems exhibiting differential affinities for a limited amount of HPr occurred, as the catabolite inhibition theory proposes. However, the results presented in this dissertation indicate that repression of synthesis rather than competitive inhibition is responsible since the lac PTS is not induced until all the glucose is exhausted. The necessity for the complete absence of glucose is supported by the definite lag period before lac PTS activity is evident in a glucose-repressed culture. Furthermore, in cells pre-induced for lactose, both glucose and lactose are utilized simultaneously (Table 22) suggesting no such competition occurs.

Catabolite inhibition may explain the results observed when fructose and lactose were present in the growth medium (Fig. 15).

The lac PTS was partially induced in the presence of fructose but did not function until fructose was exhausted; that is, lactose did not start disappearing from the medium until fructose was completely spent. However, since the lac PTS already had been induced, no diauxie occurred. These are exactly the conditions described by Thompson et al. (90) for the glucose preference over galactose in S. lactis which they explained as being due to catabolite inhibition.

Finally, most investigators believe that regulation in streptococci is the result of inducer exclusion (23,24,90), whether it be due to catabolite repression or as yet some non-defined mechanism. Catabolite repression implies the presence of cAMP and as elucidated in the Introduction, the evidence in favor of a role for cAMP in Gram-positive organisms is weak.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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